ON STIMULUS-SECRETION COUPLING IN THE EXOCRINE PANCREAS

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IN THE

EXOCRINE PANCREAS

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Hodei mihi, cras tibi

Ter nagedachtenis aan mijn ouders

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CONTENTS

CHAPTER 1

SECRETORY MECHANISMS OF THE EXOCRINE PANCREAS

1.1. HISTOLOGY

The mammalian pancreas consists for the largest part of exocrine tissue. Only a few percent of the cell mass, the so-called islets of Langerhans, have an endocrine function, viz. the secretion of insulin and glucagon.

The exocrine part of the organ maintains a basal secretion of fluid and electrolytes and of digestive enzymes, which after a suitable physiological stimulus is greatly increased. This so-called pancreatic juice is secreted into the small intestine. In most species this occurs through a single main duct. This main duct is the continuation of a branch-work of smaller ducts and ductules, of which the smallest ones originate in the acini. The morphological organisation of the exocrine pancreatic tissue has been elucidated in considerable detail by electron-microscopic studies (Ekholm and Edlund, 1959; Robertson, 1961; Sjöstrand and Elfvin, 1962; Kern and Ferner, 1971).

The lumen of the acini is surrounded by a group of acinar cells, which are characterised by the presence of large numbers of round bodies, the zymogen granules, and a highly developed endoplasmatic reticular system and Golgi-apparatus.

In the cell-type, which border the ductules, the zymogen granules are completely missing, while the endoplasmatic reticulum and Golgi-complex are much less developed than in the acinar cells. A few cells of the acini located close to

the ductule, the centroacinar cells, are morphologically very similar to the ductular cells, and share with these the presence of a large number of mitochondria, which are relatively scarce in the acinar cells (Ridderstap, 1969).

1.2. INNERVATION

The autonomic innervation of the exocrine pancreas is both sympathetic and parasympathetic, as it derives from the vagus and the splanchnic nerves, which supply the secreting cells with fibers controlling the secretion. However, the importance of the splanchnic nerve in secretion control is doubtful (Thomas, 1967).

1.3. NEURAL AND HORMONAL CONTROL OF PANCREATIC EXOCRINE SECRETION

Pancreatic exocrine secretion is under a tight neurohumoral and hormonal control. Three control phases can be distinguished: the cephalic, gastric and intestinal phases (Harper, 1972).

The cephalic phase is a vagal phenomenon and is accompanied by an increased secretion of enzymes and in some species also of fluid. This was shown by sham-feeding experiments in dog and man, in which an increased enzyme secretion was observed (Preshaw et al., 1966; Sarles et al., 1968). This effect can be mimicked by vagal stimulation (Hickson, 1970; Brown et al., 1967; Lenninger and Ohlin, 1971; see also Harper, 1967). The effect is mediated by cholinergic fibers on enzyme secretion only, since the stimulation of bicarbonate and water secretion is not blocked by atropine (Brown et al., 1967; Lenninger and Ohlin, 1971).

The gastric phase probably exists of two different effects. First, distention of the stomach causes increased

secretion of fluid and electrolytes. This involves a vago-vagal reflex pathway, since the effect is blocked by cutting the vagus nerve (White et al., 1960; Harper et al., 1959). The second effect is that mechanical stimulation of the anthral pouch causes increased enzyme secretion in cats, which is blocked by atropine, but not by section of the vagus (Blair et al., 1966). This can be explained with stimulation of a local cholinergic pathway, which causes release of gastrin. The gastrin, in turn, can cause enzyme secretion by the pancreas (Grossman, 1967).

The third phase is called the intestinal phase and is mediated by hormones. The pancreatic response is elicited by the passage of the acid and partly digested food from the stomach to the small intestine. Acidification of the small intestine causes release of secretin from intestinal stores to the blood (Grossman, 1971). The hormone stimulates the pancreas to secrete fluid and electrolytes. Passage of digestion products in a similar manner causes release of pancreozymin, which elicits enzyme secretion by the pancreas (Wang and Grossman, 1951). It is possible, that both hormones modify the action of the other (Grossman, 1970).

The effects of nerve stimulation or passage of food through the intestine can be mimicked by the administration of the gastrointestinal hormones or cholinergic agents in vivo and in vitro (Preshaw, 1967). The effect of cholinergic agents, but not that of pancreozymin, can be blocked by administration of an anticholinergic agent like atropine. Atropine does not block enzyme release from unstimulated rat or guinea pig pancreas slices (Kramer and Poort, 1972; Jamieson and Palade, 1971b). This suggests that enzyme secretion in the resting pancreas is not under neural or

hormonal control.

1.4. SECRETION OF DIGESTIVE ENZYMES

It is now quite certain that the site of enzyme secretion is in the acinar cells (Jamieson and Palade, 1967a, 1967b, 1968a, 1968b; Ichikawa, 1965). The protein molecules are synthesized at polysomes attached to the endoplasmatic reticulum, whereafter they are concentrated in smooth-surfaced vacuoles in the Golgi-complex. These condensing vacuoles are converted into zymogen granules. The mature zymogen granule moves to the apex of the cell, where its membrane fuses with the plasma membrane and becomes continuous with it. This allows the contents of the granule to be released into the lumen of the acinus. This process is often called "reverse pinocytosis" or "emiocytosis". In the resting state of the pancreas the rate of emiocytosis is low, so that mature zymogen granules accumulate in the apex of the cell.

After a physiological stimulus the rate of release of stored enzyme proteins is increased. The morphological picture of the acinar cell is changed, and zymogen discharge is accelerated within a few minutes. After 20 - 30 minutes almost no zymogen granules are left inside the cell (Palade, 1959; Ichikawa, 1965). If the stimulus is maintained, proteins continue to be packed inside condensing vacuoles, but there is no time for the completion of maturation to zymogen granules. However, the stimulus only affects the rate of transport and discharge, but does not influence the rate of synthesis of the proteins at the polysomes. This follows from the observation that in vitro incorporation of labeled amino acids in secretory proteins

is not increased in the presence of pancreozymin or acetylcholine (Hokin, 1953; Siekevitz and Palade, 1958; Webster and Tyor, 1966; Kramer and Poort, 1968; Jamieson and Palade, 1971b). The same is true for the incorporation of labeled amino acids in microsomal membrane proteins (Meldolesi and Cova, 1971) or RNA (Yang and Dickman, 1966). Neither does inhibition of protein synthesis by cycloheximide seem to affect protein discharge (Kramer and Poort, 1972; Jamieson and Palade, 1971a). Other investigators, using in vivo systems in short term experiments, have reported that stimulation in zymogen discharge rate is accompanied by stimulation of protein synthesis (Morisset and Webster, 1970; Reggio et al., 1971).

An important metabolic event accompanying stimulation of enzyme secretion should be mentioned: the so-called "phospholipid effect". Radioactive phosphate incorporation into pancreatic phospholipids is greatly enhanced by pancreozymin and acetylcholine (Hokin, 1953; Hokin and Hokin, 1955). However, the phospholipid effect can be separated from the enzyme discharge process, since Hokin (1966) observed with pigeon pancreas slices that upon omission of Ca^{2+} from the medium the release of enzymes after addition of a stimulant was decreased or even stopped, but the increase in ^{32}P-phosphate incorporation into phospholipids persisted.

Energy requirements

The energy requirements of the cellular events under neural and hormonal control were studied by Jamieson and Palade (1971a). They showed that for the discharge of enzymes by guinea-pig pancreas slices a continuous supply of

ATP from respiratory pathways is necessary. Discharge in the presence of cycloheximide, an inhibitor of protein synthesis, was blocked by addition of inhibitors of oxidative phosphorylation, but not by addition of glycolytic inhibitors. There was no evidence that energy is needed for the conversion of condensing vacuoles into zymogen granules in the Golgi region. The Na-K activated ATPase cation pump system is not involved in enzyme discharge, because ouabain does not inhibit enzyme secretion in vitro (Ridderstap and Bonting, 1969a; Jamieson and Palade, 1971a).

Stimulus-secretion coupling

The question may now be raised how the extracellular stimulants acetylcholine or pancreozymin exercise their effects intracellularly, so that discharge of exportable enzymes is effected. In many secretory processes cyclic AMP has been shown to be an intermediate, as in amylase secretion by the parotid gland (Bdolah and Schramm, 1965; Schramm and Naim, 1970), insulin secretion by the endocrine cells of the pancreas (Sussman and Vaughan, 1967; Turtle and Kipnis, 1967), secretion of trophic hormones by the anterior pituitary (Fleischer et al., 1969; Lockhart Ewart and Taylor, 1971; Zor et al., 1969; Ratner, 1970; Lemay and Labrie, 1972) and secretion of thyroid hormones by the thyroid gland (Neve and Dumont, 1970; Tonoue et al., 1970).

It is only logical that attempts should be made to establish whether this nucleotide plays a similar role in pancreatic enzyme secretion. At the start of our work only three reports dealing with this subject were available. Kulka and Sternlicht (1968) had reported that cyclic AMP and its mono- and dibutyrylderivatives induce enzyme

secretion from mouse pancreas slices. This effect was mimicked by theophyllin, which inhibits the enzymatic breakdown of cyclic AMP. Ridderstap and Bonting (1969c) had obtained similar results with the isolated rabbit pancreas. Addition of cyclic AMP or theophyllin in millimolar concentrations to the bathing fluid caused stimulation of enzyme secretion, while theophyllin potentiated the stimulating effect of pancreozymin. Case et al. (1969) also observed stimulation of enzyme secretion in the isolated perfused cat pancreas by cyclic AMP and theophyllin. However, these authors ascribed this phenomenon to a washout effect, caused by the simultaneously observed stimulation of fluid secretion.

On the basis of their observations Ridderstap and Bonting (1969c) put forward a hypothesis for the stimulus-secretion coupling in the process of pancreatic enzyme secretion. A scheme of the reactions which would play a role in this process is shown in Fig. 1. Pancreozymin would exert its action by activating adenylate cyclase, the enzyme which catalyzes the formation of cyclic AMP from ATP. The nucleotide in turn would activate phospholipase A, known to be present in an inactive form in pig pancreas zymogen granules (De Haas et al., 1968b). This would lead to a local formation of lysophospholipids, which by their known lytic action on phospholipid membranes (Gottfried and Rapport, 1963) would cause membrane fusion. Most of the work described in this thesis concerns experiments aimed at testing the validity of this hypothesis. Since there is no evidence for a second messenger role of cyclic AMP in the action of acetylcholine, the hypothesis cannot offer an explanation for the mechanism of action of this stimulant of

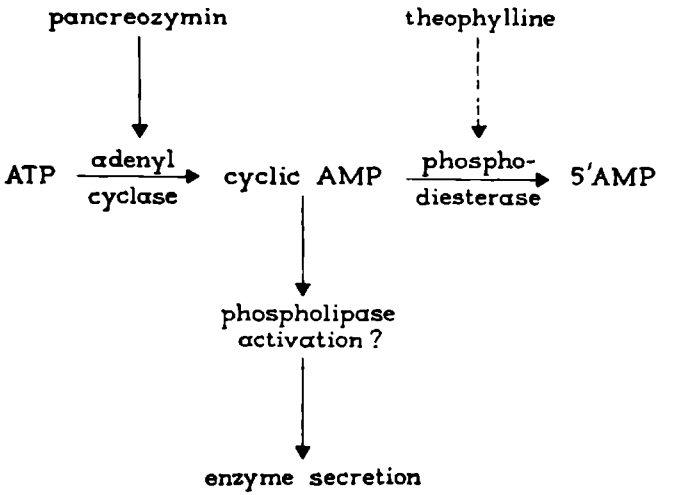

Fig. 1. Schematic diagram of the role of cyclic AMP in pancreatic enzyme secretion, as proposed by Ridderstap and Bonting(1969c).

pancreatic enzyme secretion.

There is increasing evidence that in many secretory processes, in which cyclic AMP is found to act as a second messenger, movements of Ca^{2+} may play an important role in the coupling mechanism. Changes in Ca^{2+} concentrations inside the cell or of intracellular compartments may affect enzymatic processes within the cell or influence physical parameters like membrane permeability (Rubin, 1970). Such changes may also interfere with the formation of cyclic AMP or with the intracellular effects of this nucleotide (Rasmussen, 1970). For the exocrine pancreas it is interesting that, as stated before, extracellular Ca^{2+} is necessary for stimulation of enzyme secretion and that the Ca^{2+} concentration in pancreatic juice is correlated with

the concentrations of the secretory enzymes in dog pancreas in vivo (Zimmerman et al., 1967).

Electrical phenomena observed to occur in the acinar pancreatic cell in response to a stimulus also suggest a role of Ca^{2+} in enzyme secretion. Pancreozymin and acetylcholine are reported to cause a depolarisation of the mouse pancreatic acinar cell, suggesting an increased permeability for cations (Dean and Matthews, 1972; Petersen and Matthews, 1972). In contrast, in the rat pancreatic acinar cell, both agents were reported to cause a slight hyperpolarisation (Kanno, 1972), Whether these phenomena bear a causal relationship with enzyme secretion remains to be established.

1.5.SECRETION OF FLUID AND ELECTROLYTES

The mammalian pancreas secretes a fluid, which is isotonic with the extracellular fluid independent of the flow rate. There is still no complete certainty as to the cells responsible for fluid and electrolyte secretion. The available evidence points to the epithelial cells in the intercalary and intralobular ducts, the ductular and centroacinar cells. Ethionine, which causes destructive lesions in the acinar cells, but not in the ductular cells, inhibits enzyme secretion, but does not affect fluid secretion (Kalser and Grossman, 1954). In contrast alloxan, which damages ductular cells, lowers the response to secretin stimulation (Grossman and Ivy, 1946). Furthermore, Ridderstap (1969) observed dilatation of the intercellular spaces between centroacinar and ductular cells in response to secretin, which phenomenon is comparable to similar morphological changes observed in reabsorbing gall bladder

(Kaye et al., 1966) and other transporting epithelia.

It has long been known that the sodium and potassium concentrations in pancreatic juice are approximately the same as in plasma (Ball and Johnston, 1930). Concentrations of the divalent cations Ca^{2+} and Mg^{2+} are lower than in plasma (Dreiling and Janowitz, 1956). Sodium is readily transported from plasma to secreted fluid. Extracellular ^{22}Na appears after a few minutes in juice, in vivo (Montgomery et al., 1941) as well as in vitro (Ridderstap and Bonting, 1969a).

Bicarbonate and chloride are the principal anions in pancreatic juice. While the sum of their respective concentrations in the juice is equivalent with that of the cations, the ratio between them is a function of flow rate, bicarbonate concentration rising with increasing flow rate. This was observed in vivo (see Janowitz, 1967) as well as in vitro (Case et al., 1968; Ridderstap, 1969). This led to the hypothesis, that the primary secretion originates from the centroacinar cells and is rich in bicarbonate, which exchanges with chloride during its passage through the ducts (Case et al., 1968).

Energy requirement

Fluid and electrolyte secretion is dependent on respiratory energy in the form of ATP, which activates the sodium pump, Na-K-activated ATPase. This was shown by Ridderstap and Bonting, 1969a), who observed excellent parallelism between the inhibition of flow rate and of pancreatic Na-K-activated ATPase by ouabain in rabbit pancreas. Furthermore, the flow rate is linearly dependent on the sodium concentration in the perfusion or bathing fluid (Case et al., 1968;

Ridderstap, 1969).

Stimulus-secretion coupling

There is little known about the mechanism, by which secretin increases the fluid secretion. When our experiments were in progress, there were only few reports dealing with a role of cyclic AMP in fluid secretion. Case et al. (1969) reported that dibutyryl cyclic AMP (1 mM) and theophylline (0.4 mM) increase fluid secretion by the isolated perfused cat pancreas. Ridderstap (1969) did not study the effects of cyclic AMP or its dibutyryl derivative on fluid secretion by the isolated rabbit pancreas, since this preparation shows only little stimulation by secretin, which makes it rather unsuitable for the study of this aspect of exocrine pancreatic secretion.

CHAPTER II

EFFECTS OF VARIOUS AGENTS ON FLUID AND ENZYME SECRETION BY THE ISOLATED RABBIT PANCREAS

2.1. INTRODUCTION

In Chapter I the existing evidence for an intermediary role of cyclic AMP in exocrine pancreatic secretion has been discussed. There were only few observations favoring a role of the nucleotide in enzyme secretion, and there was also some evidence to the contrary. There is clearly a need for more information.

Two different approaches are possible to establish whether cyclic AMP is indeed an intermediate. First one can try to determine whether in response to a stimulus the nucleotide concentration in the cell is raised. The results of this approach will be discussed in Chapter III. Secondly, one can investigate whether the nucleotide added exogenously is able to mimick the effects of the physiological stimuli.

This chapter will consider experiments of the latter type. In this approach it is best to use an isolated or perfused organ. The isolated rabbit pancreas, with which experience was available in our laboratory, was chosen as the experimental system. The previous experiments of Ridderstap and Bonting (1969c) have been repeated, since the observed effects of added cyclic AMP were small compared to the response to acetylcholine and pancreozymin and were only significant for one parameter, total protein secretin, and then

only with high concentrations (1 mM) of the nucleotide.

Another possibility is to investigate the effects of agents, which in several tissues are known to increase cyclic AMP production, e.g. high extracellular potassium concentration and inhibitors of cyclic AMP phosphodiesterase, the enzyme which breaks down cyclic AMP. In addition to the enzyme secretion, we have dètermined the effects on flow and electrolyte secretion.

2.2. METHODS

Preparation of the isolated pancreas preparation

New Zealand white rabbits, weighing 2-3 kg, are fasted 24 hours before the experiment and killed by a blow on the neck and bleeding from the carotis. The pancreas is prepared according to Rothman and Brooks (1965) with only a slight modification. The mesomential loop is cut from the rest of the intestine, leaving a small part of the rectum attached to the omentum in order to prevent damage to the omentum. Since the pancreas is at some places in very close contact with the stomach, a small part of the stomach is also left attached to the duodenum. The mesomental loop is further isolated by cutting it away from the mesentery. The open ends of the duodenum and rectum are closed by tying them with surgical suture. The entire preparation is mounted on a polyvinylchloride frame (20 x 10 cm) by tying various places of the intestinal wall to holes in the frame. The main pancreatic duct is opened as closely as possible to the duodenum and cannulated with polythene tubing (0.57 mm inner diameter). After lowering the frame into a plexiglass chamber (20 x 2 x 10 cm), the tube is passed through a

hole in the bottom of the chamber. The organ is bathed in 300 ml of a balanced salt solution, which is continuously gassed with carbogene (95% O_2, 5% CO_2), while the temperature is maintained at 37°C by circulating water of that temperature through the chamber wall. Continuous mixing of the bathing medium is accomplished by admitting gas through a perforated glass tube placed at the bottom of the chamber. The entire procedure after killing of the rabbit takes approximately 10 minutes. The secreted fluid is collected in pre-weighed glass tubes (8mm inner diameter and 6cm height).

The composition of the normal bathing solution is as given in Table I.

TABLE I

COMPOSITION OF THE BATHING MEDIUM

Na^+	170 mM
K^+	4.9 mM
Ca^{2+}	2.5 mM
Mg^{2+}	1.2 mM
HCO_3^-	25 mM
$H_2PO_4^-$	1.2 mM
Cl^-	156.1 mM
glucose	5.5 mM

In the experiments, in which the effect of a high potassium environment is examined, the solution is modified by partly

replacing NaCl by an equivalent amount of KCl (up to 120 mM). Before incubation the pH of the solution is adjusted to 7.2 by addition of hydrochloric acid. By gassing with carbogene this pH can be maintained during incubation.

Electrolyte determinations

Sodium and potassium concentrations are measured by flamephotometry on an Eppendorf flamephotometer. The juice is diluted with distilled water to an expected concentration of sodium between 0.2 and 1.0 mM, and if necessary for potassium to an expected concentration of 0.02 to 0.10 mM. Standard solutions of sodium and potassium in the same range are used for calibration curves, which are virtually linear in these concentration ranges.

Protein determination

Protein is determined according to Lowry et al. (1951) with bovine serum albumin (Behringwerke) serving as a standard.

2.3. RESULTS

Basal secretion

Table II shows the basal secretion of fluid and protein by the isolated rabbit pancreas. Flow increases somewhat during the first hour, being relatively constant during the second, third and fourth hours, and thereafter decreases again. Protein secretion is large during the first hour, but thereafter slows down, although not becoming completely constant. The values in the table are in the same range as those reported by Rothman (1964) and Ridderstap and Bonting

TABLE II

BASAL SECRETION BY THE ISOLATED RABBIT PANCREAS

Protein is measured in the fluid, which is collected in hourly periods. Averages of 5 experiments are given with the S.E. of the mean.

number of hourly period	flow, µl/hr	protein, mg/hr
1	351 ± 49	5.61 ± 1.13
2	398 ± 60	1.85 ± 0.28
3	394 ± 51	1.48 ± 0.35
4	363 ± 49	1.18 ± 0.21
5	325 ± 42	1.04 ± 0.21

(1969a). The former author reports that the flow rate of the organ in vitro is up to ten times larger than in vivo. In vitro flow is only slightly increased by secretin added to the bathing medium (Rothman, 1964; Ridderstap, 1969), and the system is therefore not very suitable for studies of stimulation of the fluid secretion. Observations by Hubel (1970) suggest that this is due to the removal of a net α-adrenergic tone, which has an inhibitory effect on fluid secretion in vivo. We have tested, whether addition of α-adrenergic substances to the bathing medium will reduce the in vitro flow to the rates observed in vivo in order to make the system suitable for in vitro studies of flow stimulation. However, addition of 5×10^{-5}M isoprenaline or adrenalin after the second hour reduces the flow rate only by maximally 30%. Addition of secretin (330 U per 1) after the third hour still increases the flow rate by only 30%, about the same as in the absence of adrenergic agents.

Effects of pancreozymin and acetylcholine

Table III shows the effect of pancreozymin (33 U/l) and acetylcholine (10^{-5}M), added after the second hour. While the flow rate does not change significantly, both agents greatly increase the protein output. This is in accordance with the findings of Rothman (1964) and Ridderstap (1969).

Effects of inhibitors of cyclic nucleotide phosphodiesterase

When cyclic AMP is the intermediate in the action of a hormone, compounds which inhibit its breakdown usually mimick the effect of the stimulant. Therefore we have studied the effects on protein secretion of theophylline and

TABLE III

EFFECTS OF PANCREOZYMIN AND ACETYLCHOLINE ON SECRETION BY THE ISOLATED RABBIT PANCREAS

Protein is measured in the juice, which is collected in hourly periods. Pancreozymin or acetylcholine are added in the stated concentrations after the third hour. Results are given as percentage change relative to the third hour.

Substance added	flow, change in % exp.1	exp.2	aver.	protein, change in % exp.1	exp.2	aver.
Pancreozymin (33 U/l)	- 15	- 10	- 12	+ 357	+ 453	+ 405
Acetylcholine (10^{-5}M)	- 17	- 27	- 22	+1148	+ 943	+1046

papaverine, which are inhibitors of cyclic AMP phosphodiesterase. Theophylline, added after the third hour in concentrations up to 10 mM, does not cause stimulation of enzyme secretion. This is in contrast with the findings of Ridderstap and Bonting (1969c), who observed a significant (82%) stimulation of protein secretion. On the other hand, papaverine, added after the third hour in 1 mM concentration, significantly stimulates the protein output while simultaneously reducing the flow rate. Results are shown in Table IV, while Fig. 2 shows a representative experiment with papaverine.

Effect of cyclic nucleotides

Cyclic AMP, added after the third hour in a concentration of 1 mM, does not significantly influence either flow rate or protein secretion in the subsequent hours. Dibutyryl cyclic AMP in a concentration of 0.5 mM has no effect either. Moreover, a combination of 1 mM cyclic AMP and 1 mM papaverine causes no greater stimulation than papaverine alone. Cyclic GMP, the guanosine analogue of cyclic AMP, has no influence on secretion by the isolated organ when added in a concentration of 0.4 mM. The results of these experiments are shown in Table IV.

Effects of high potassium concentrations in the bathing medium

Bdolah et al. (1964) have shown that enzyme secretion by rat parotid stices is stimulated, when the extracellular potassium concentration is increased. Rasmussen and Tenenhouse (1968) have found that this is accompanied by an increased intracellular cyclic AMP concentration. Therefore

TABLE IV

EFFECTS OF PHOSPHODIESTERASE INHIBITORS AND CYCLIC NUCLEOTIDES ON SECRETION BY THE ISOLATED RABBIT PANCREAS

Protein is measured in the fluid, which is collected in hourly periods.
All agents are added at the end of the third hour in the final concentration shown.
Results are given as percentage change relative to the third hour and are in the case of papaverine given with S.E. of the mean.

Substance added	number of experiments	flow, change in %	protein, change in %
Theophylline (10^{-2}M)	2	- 10, 0	- 30,- 17
Papaverine (10^{-3}M)	5	- 27 $\pm$ 21	+275 $\pm$ 77
Cyclic AMP (10^{-3}M)	2	- 10,- 7	+ 58, 0
Cyclic GMP ($4x10^{-4}$M)	1	- 4	- 42
Cyclic AMP (10^{-3}M) + papaverine (10^{-3}M)	2	- 23,- 44	+122,+213

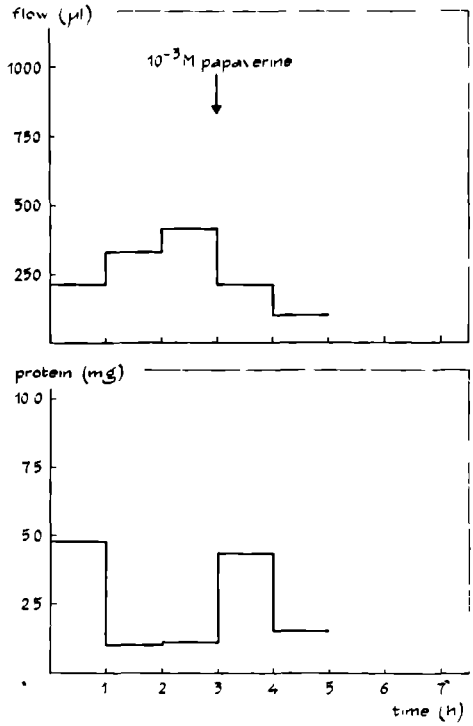

Fig. 2. Effect of papaverine on secretion by the isolated rabbit pancreas. Protein is measured in the juice, which is collected in hourly periods. Papaverine is added at the end of the third hour.

the effects of high potassium concentrations in the bathing fluid have been studied. Table V shows results with 75 and 120 mM potassium present in the medium. In the presence of 75 mM K^+ protein secretion is clearly stimulated, while the flow rate is somewhat decreased. Increasing the potassium concentration to 120 mM does not further enhance protein

TABLE V

EFFECT OF HIGH.POTASSIUM CONCENTRATIONS IN THE BATHING MEDIUM ON SECRETION BY THE ISOLATED RABBIT PANCREAS

Protein is measured in the fluid, which is collected in hourly periods. At the end of the second hour the bathing medium is replaced by a medium, in which NaCl is partly replaced by an equivalent amount of KCl. In two experiments atropine is added. Results are expressed as percentage change, relative to the second hour and are averages of a given number of experiments. If possible, they are given with S.E. of the mean.

Bathing medium	number of experiments	flow, change in % 3d hour	4th hour	protein, change in % 3d hour	4th hour
75mM K^+, 100 mM Na^+	8	-16± 4	+ 3±3	+308±106	+201±73
120 mM K^+, 55 mM Na^+	3	-55±13	-60±9	+184± 32	+142±95
75 mM K^+ + 10^{-5}M atropine	2	-32,-4	-18,+1	-42, -20	-50,-26

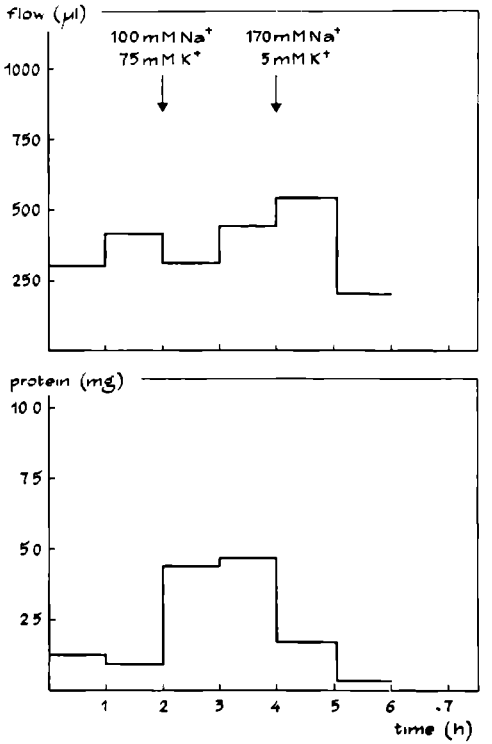

Fig. 3. Effect of a high potassium concentration in the bathing medium on the secretion of the isolated rabbit pancreas. Protein is measured in the fluid, which is collected in hourly periods. At the end of the second hour the medium is replaced by one in which NaCl is partly replaced by an equivalend amount of KCl. At the end of the fourth hour the medium is replaced by the original medium.

secretion, but causes a pronounced inhibition of fluid secretion. In both cases the effect on fluid secretion levels off after one hour. When after two hours the high potassium medium is replaced by the normal bathing medium, the flow rate rises sharply to values larger than before stimulation. In Fig. 3 a representative experiment is shown.

We have tested whether the fluid secretion, left after the addition of a high potassium medium is still sensitive to ouabain. This proves to be the case, since 10^{-4}M ouabain, which blocks both basal and stimulated fluid secretion of the isolated rabbit pancreas almost completely (Ridderstap and Bonting, 1969a), further decreases the fluid output in high potassium media.

Since there is evidence (Argent et al., 1971) that the effect of high extracellular potassium concentrations is due to the release of acetylcholine, we have tested the effect of addition of atropine. When 10^{-5}M atropine is added to the medium containing 75 mM potassium, the increase in protein secretion is abolished, but the reduction in fluid volume remains (Table V).

Effect of high potassium concentration on ion concentrations in fluid

When the sodium and potassium concentrations in the fluid are monitored, it is observed that after changing the medium potassium concentration, these concentrations always become equal to those in the bathing medium after some time. Fig. 4 shows the results of a representative experiment.

Effects of colchicine and vincristine on protein secretion

Colchicine or vincristine, added at the end of the sec-

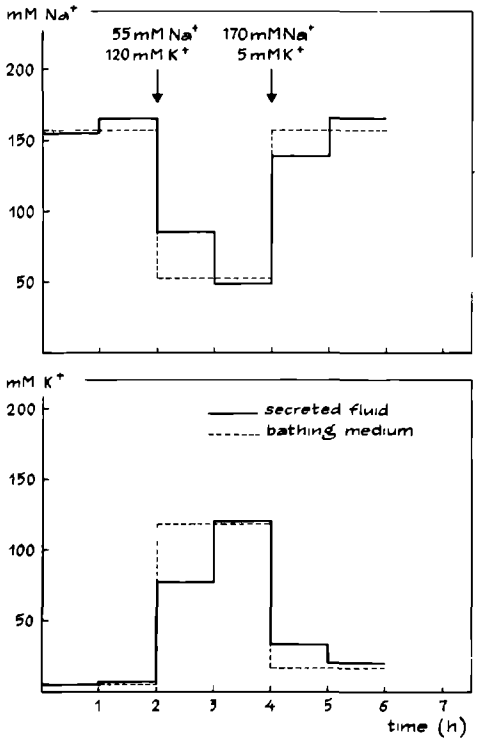

Fig. 4. Effect of changes in sodium and potassium concentrations in the bathing medium on their concentrations in the fluid secreted by the isolated rabbit pancreas. Sodium and potassium concentrations are measured in the fluid, which is collected in hourly periods. At the end of the second hour the medium is changed for one in which NaCl is partly replaced by an equivalent amount of KCl. At the end of the fourth hour the original medium is returned. Solid and broken lines indicate the concentrations, measured in secreted fluid and bathing medium, respectively.

ond hour of incubation, in concentrations of up to 10^{-4} M and 10 µg per ml respectively, do not inhibit stimulation of protein secretion by 10^{-5} M acetylcholine or 33 U per liter pancreozymin, added at the end of the third hour.

2.4. DISCUSSION

Several investigators have examined whether cyclic AMP, one of its butyryl derivatives,or cyclic AMP phosphodiesterase inhibitors are able to stimulate enzyme secretion by pancreas preparations in vitro. Positive as well as negative results have been obtained. In mouse pancreas fragments a clear stimulation has been found with all these compounds (Kulka and Sternlicht, 1968). Ridderstap and Bonting (1969c) observed a 60% stimulation of protein secretion in the isolated rabbit pancreas by 1 mM cyclic AMP in the second hour after addition of the nucleotide. Theophylline (10 mM) caused a 80% stimulation in the first hour after addition and also potentiated the action of a submaximally stimulating concentration of pancreozymin. These effects are, however, small compared to the more than ten-fold stimulation, maximally obtainable with pancreozymin or acetylcholine. In the same preparation Knodell et al. (1970) have observed a very small increase in chymotrypsin and trypsin secretion by 0.01 mM dibutyryl cyclic AMP and also by 10 mM theophylline. Amylase secretion by rat pancreatic fragments is stimulated by 1 mM dibutyryl cyclic AMP to approximately the same extent as by pancreozymin and urecholine (Morisset and Webster, 1971). In the same preparation Bauduin et al. (1971) have observed stimulation of amylase secretion by 10 mM cyclic AMP or a 10-fold lower concentration of its dibutyryl derivative. The maximally

obtainable stimulation by these compounds is only about one third of the maximal effect of the physiological stimulants. The effect of the nucleotide is doubled by 10 mM theophylline which by itself elicits no response. Release of radioactive protein from rat pancreas fragments, preloaded with [^{3}H]-leucine, is increased by 1 mM dibutyryl cyclic AMP (Heisler et al., 1972), but the effect is only a third of that of a maximally stimulating concentration of carbachol.

In pieces or slices of guinea pig pancreas cyclic AMP or its dibutyryl derivative and also theophylline do not elicit any stimulatory response (Jamieson and Palade, 1971a; Benz et al., 1972). In the perfused cat pancreas a slight increase in amylase output by 1 mM dibutyryl cyclic AMP has been observed by Case and Scratcherd (1972), but these authors suggest that this effect may be due to wash-out by the simultaneously strongly increased fluid secretion.

In the isolated rabbit pancreas we are not able to confirm the findings of Ridderstap and Bonting (1969c). Both cyclic AMP and its dibutyryl derivative as well as theophylline are without effect on protein secretion. This has led us to test the effect of papaverine, which is a far stronger inhibitor of cyclic AMP breakdown as theophylline. This compound in a 1 mM concentration clearly stimulates protein secretion, while fluid secretion is strongly inhibited. The effect is lower than that seen in the presence of physiological stimulants.

The lack of effect of cyclic AMP in our experiments can be explained in two ways. First, it is possible that the intracellular concentration of the nucleotide is not a rate-limiting factor in the process of enzyme secretion. Secondly, however, it is also possible that cyclic AMP

phosphodiesterase, which at least in rat pancreas is present in great abundance (see section 5.3.) prevents cyclic AMP from reaching intracellular concentrations high enough to elicit an effect. A more or less similar explanation for the divergence of the effects of theophylline and papaverine is possible. In test tube experiments with 4 µM cyclic AMP the concentration of papaverine which has been used (1 mM) causes 100% inhibition of rat pancreatic phosphodiesterase; in the presence of 10 mM theophylline, however, a substantial phosphodiesterase activity is still left (see section 5.4.). Against the explanations in favour of a mediating role of cyclic AMP could be used the observation that a combination of cyclic AMP and papaverine has no greater effect than papaverine alone.

The effect of a high concentration of potassium in the bathing medium has been studied because it has formly been shown that in rat parotid slices this causes stimulation of amylase secretion (Bdolah et al., 1964) as well as an increase in cyclic AMP content of this tissue (Rasmussen and Tenenhouse, 1968). The stimulation of amylase secretion is accompanied by a decrease in the fluid secretion. A similar effect has been found in the perfused cat pancreas (Argent et al., 1971) and in guinea pig pancreas slices (Benz et al., 1972). In confirmation of the results of these authors we find that atropine blocks the stimulation of enzyme secretion by potassium, which suggests that potassium elicits its secretory response by releasing acetylcholine from intrapancreatic stores. The potassium effect, therefore, cannot serve as evidence for a cyclic AMP involvement.

The conflicting results of our experiments and those of

other authors may, in part, be due to species differences in the sensitivity of the exocrine pancreas to exogenous cyclic AMP. Another possibility is that, whether or not cyclic AMP is an intermediate factor in the stimulus-secretion coupling process, another rate-limiting factor plays a role in this process, e.g. calcium ions (see section 8.4.). A combination of these two factors may explain the varying sensitivity of pancreatic enzyme secretion to cyclic AMP.

The effect of cyclic GMP has been tested, because acetylcholine was shown to increase levels of this nucleotide in the perfused rat heart (George et al., 1970) and in rat brain slices (Kuo et al., 1972). This effect is bound to intact cells and does not depend on a direct stimulation of a guanylate cyclase. However, a 0.4 mM concentration of this nucleotide is without effect on enzyme secretion in the isolated rabbit pancreas.

Microtubular or microfilamentous systems do not seem to be involved in enzyme secretion, since neither colchicine nor vincristime inhibits basal or stimulated protein secretion by the isolated rabbit pancreas. This confirms similar observations of Jamieson and Palade (1971b)and Benz et al. (1972), both made with colchicine in guinea pig pancreas. This is in contrast with other bulk secretion systems like pancreatic beta-cells and thyroid gland, where colchicine inhibits release of insulin (Malaisse et al., 1971) and thyroxine (Williams and Wolff, 1971), respectively.

Whereas Case and Scratcherd (1972) report a stimulating effect of cyclic AMP and phosphodiesterase inhibitors on fluid secretion by the perfused cat pancreas, we do not observe such an effect in the isolated rabbit pancreas. The latter finding may be explained by the fact that the isolated rabbit pancreas

secretes fluid at a rate, which is comparable to that of the organ in vivo, during maximal stimulation by secretin (Rothman, 1964), and which is almost insensitive to added secretin (Ridderstap, 1969).

A final comment about the effects of high potassium concentrations in the bathing medium on fluid and electrolyte secretion is in order. The fact that the concentrations of sodium and potassium in the fluid closely follow those in the bathing medium is a confirmation of the early observation of Ball and Johnston(1930), who observed in the dog pancreas that intravenous injections of hypertonic NaCl and KCl increase sodium and potassium concentration in pancreatic fluid to exactly the same level as in plasma. The effect in our experiments is fully reversible. These effects should be considered in the light of the evidence presented by Ridderstap (1969) for the involvement of a Na-coupled isotonic water transport through local osmosis. There are two effects: a. the Na^+ concentration (and also the K^+ concentration) in the fluid follows that in the bathing medium, b. the volume is reduced upon decreasing the Na^+ concentration in the bathing medium regardless of whether atropine is added. The former effect is in agreement with the local osmosis theory: water follows isotonically the Na^+ ions, which are actively transported across the cell membrane into the lumen of the duct. The latter effect was also found in the perfused cat pancreas (Argent et al., 1971) and is most likely due to a slowing down of the Na-K-ATPase activated sodium pump through a lowering of the intracellular sodium concentration. In the case of 75 mM K^+ - 100 mM Na^+ the effect is small and transient, but in the case of 120 mM K^+ - 55 mM Na^+ it is large and lasting. The reduced water flow is still

due to the (reduced) activity of the Na-K ATPase system, since it is inhibited by ouabain. The finding that the K^+ concentration also follows that in the bathing medium may be ascribed to the osmotic requirements, which have also been shown in the experiments of Diamond (1962) on the gall bladder.

CHAPTER III

ADENYLATE CYCLASE IN THE RAT PANCREAS. PROPERTIES AND STIMULATION BY HORMONES

3.1. INTRODUCTION

To establish the possible involvement of cyclic AMP in the process of pancreatic secretion, the subsequent steps in the stimulus-secretion coupling can be considered. Chapter II described experiments, in which the second step was examined. Tests were made as to whether cyclic AMP itself or compounds, known to inhibit the breakdown of the nucleotide, are able to mimick the effect of the stimulus. As the results which were gained offered conflicting evidence, it is necessary to consider the first step in the coupling process: that is, whether the stimulus gives rise to an increased formation of cyclic AMP. This can be done in living cells or in broken cell preparations. The last approach involves the study of adenylate cyclase, the enzyme which catalyses the conversion of ATP to cyclic AMP.

Adenylate cyclase is now known to consist of at least two different entities, both localized in the plasma membrane (Birnbaumer et al., 1971). The catalytic site, which is localized at the inner side of the membrane is activated by fluoride in broken cell preparations, but not in living cells. Hormones can activate adenylate cyclase by acting on a receptor site on the outer side of the membrane.

This chapter describes the study of this enzyme and its characteristics in rat pancreas. The activity is

determined by measuring the production of $[^{32}P]$-cyclic AMP from $[\alpha\text{-}^{32}P]$ -ATP as substrate. This is the most simple and direct method, which has the advantage over methods, which use substrate with another label, that little or no other radioactive products are formed (Bär and Hechter, 1969).

3.2. METHODS

Tissue preparation

Male Wistar rats, 2-3 months old with free access to food and water, are killed by a blow on the head. The pancreas is removed and placed immediately in an ice cold solution, containing 10 mM TRIS, 2.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mg/ml trypsin inhibitor at pH 7.4. After removal of fat and connective tissue, the tissue is minced and homogenized gently by hand in 4 volumes of the same solution as above, using a loosely fitting Teflon pestle. The homogenate is filtered through surgical gauze and centrifuged at 4000 g_{max} for 10 minutes at $0^{o}C$. The pellet is washed twice with 5 volumes and then suspended in 3 volumes of the same solution. The resulting suspension is called "enzyme preparation". It is used immediately for enzyme assay, since storage of the preparation at $0^{o}C$ or in the frozen state always leads to decreased activities.

Adenylate cyclase assay

The incubation medium is prepared by mixing equal volumes of the following solutions: 0.4 M TRIS-HCl (pH 7.4); 50 mM $MgCl_2$; 0.1 M theophylline; 50 mM phosphoenolpyruvate trisodium salt; 2 mg/ml pyruvate kinase in 40 mM TRIS-buffer pH 7.4; 4 mM ATP containing 0.2×10^6 - 10^6 CPM/µl as

[α-^{32}P] ATP. To this mixture a double volume of water, in which any further reagents are dissolved, is added. After preincubation of 40 µl of this medium in microtest tubes for 3 minutes at 37°, the reaction is started by the addition of 10 µl enzyme preparation. Thus, the following final concentrations are maintained: TRIS-HCl, 46 mM; ATP, 0.4 mM; Mg^{2+}, 5.5 mM; EDTA , 0.5 mM; theophylline, 10 mM; phosphoenolpyruvate, 5mM; pyruvate kinase, 0.2 mg/ml; trypsin inhibitor, 0.4 mg/ml; final pH 7.4. Unless otherwise stated, incubations are carried out at 37° for 15 minutes. The reaction is terminated by placing the tubes for 2 minutes in boiling water, where-upon they are placed in ice; 5 µl of a solution of cyclic AMP (5 mg/ml in water) is then added and the tubes are centrifuged.

The separation of the radioactive product from the substrate and from other radioactive metabolites is carried out by thin-layer chromatography (TLC) according to Woods and Waitzman (1970). A 30-µl aliquot from the clear supernatant of each tube is placed on TLC-medium (Chromar-Sheet 500). After developing the thin layers with a mixture of 2-propanol, ethylacetate and 13 N ammonia (59-25-16), the cyclic AMP spots are visualized under 254 nm UV light. This development system leaves other adenosine phosphates (ATP, ADP and AMP) and also inorganic phosphate and pyrophosphate on the starting point, while cyclic AMP has an Rf of approximately 0.40. The spots are then cut out and placed in a scintillation vial. After addition of 10 ml Bray scintillation solution radioactivity is counted in a liquid scintillation spectrometer (Packard Tri-Carb; model 3380). The amount of cyclic AMP formed during the incubation is calculated from the

specific activity of $[\alpha\text{-}^{32}P]$-ATP in the reaction mixture and the amount of cyclic $[^{32}P]$-AMP formed minus the reaction blank. The blank after proper development of the thin layer is normally about 0.01% of the radioactivity of the initially present ATP. All determinations are performed in duplicate and the results, which usually agree within 5%, averaged. Enzyme activity is expressed in pmol cyclic AMP formed per mg protein per minute of incubation time. Protein is measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

3.3. RESULTS

Basal and fluoride stimulated activity

Basal activity of the enzyme in the various preparations is low and ranges from undetectable ($<$ 0.5 pmol/min/mg protein) to about 3 pmol/min/mg protein.

Sodium fluoride has a strongly activating affect on the enzyme. Fig. 5 shows the activity as a function of the fluoride concentration. Maximal activity occurs at 10-40 mM NaF. The fluoride (10 mM) stimulated activity of the various enzyme preparations ranges from 15-40 pmol/min/mg protein. The possible cause of this variability will be discussed in section 3.4. The 4000 x g particulate fraction contains about 2/3 of the total pancreatic enzyme activity.

Properties of the NaF stimulated enzyme

The very low basal activity of the enzyme makes it impossible to determine the properties of the enzyme in the non-stimulated condition. Hence, we have chosen to study

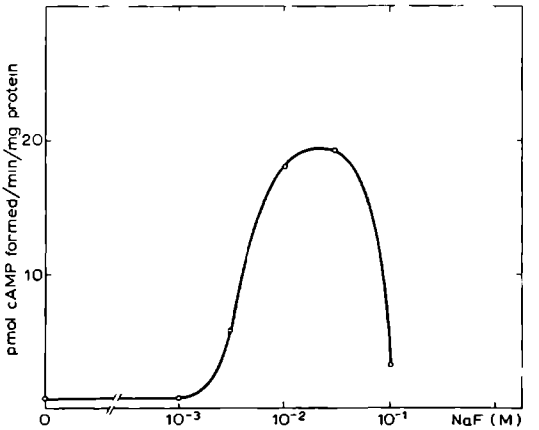

Fig. 5. Effect of fluoride on adenylate cyclase activity. Freshly prepared enzyme preparation is incubated for 15 min with varying concentrations of NaF.

these properties in the presence of 10 mM NaF. The activity is constant during 30 min of incubation under control conditions (Fig. 6). A linear relationship between cyclic AMP formation and enzyme concentration exists up to 150 µg protein, the highest enzyme concentration used (Fig.7). Hence, all further experiments have been carried out with 10- or 15- minute incubation periods and with less than 150 µg protein to ensure linear assay conditions.

Fig. 8 represents a Lineweaver-Burk plot for the relation between enzyme activity and substrate concentration. The value for K_m is approximately 0.3 mM ATP. A concentration of 0.4 mM ATP has been used in all subsequent enzyme assays. Although this concentration is sub-optimal, there are valid reasons for this choice: firstly, the need to maintain a high specific radioactivity of the substrate

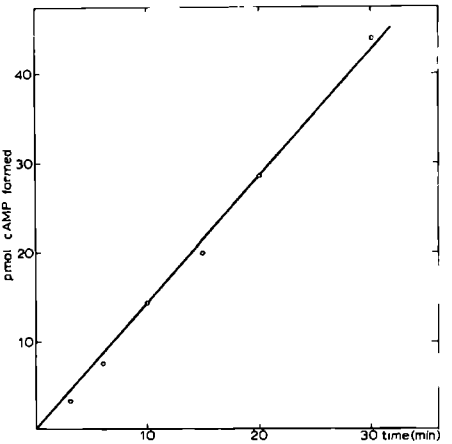

Fig.6. Linearity of fluoride stimulated cyclic AMP formation with respect to incubation time. Freshly prepared enzyme preparation (8 µg protein) is incubated in the presence of 10 mM NaF.

and thus of the radioactive cyclic AMP formed, and secondly the occurrence of substrate inhibition at ATP concentrations above 1.5 mM.

The relation between enzyme activity and pH of the incubation medium is shown in Fig.9. The pH-optimum of the NaF stimulated adenylate cyclase activity is 7.4.

Fig. 10 shows the effect of varying Mg^{2+} concentrations on the fluoride stimulated activity. The rate of cyclic AMP formation reaches a maximal value at a concentration of 5 mM Mg^{2+}. When no Mg^{2+} is added, some residual activity is left, which can be abolished by addition of 1 mM EDTA. This suggests that the residual activity in the absence of

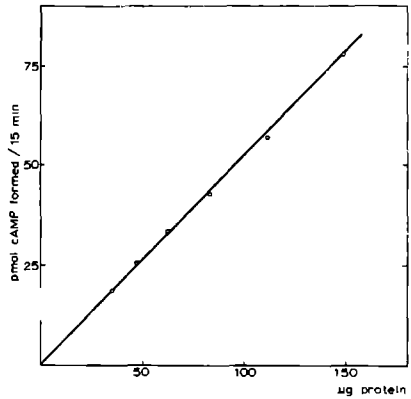

Fig. 7. Linearity of fluoride stimulated cyclic AMP formation with respect to enzyme concentration. Dilutions of freshly prepared enzyme preparation are incubated for 15 min in the presence of 10 mM NaF.

added Mg^{2+} is due to the presence of some bivalent cation in the enzyme preparation.

Some incubations have been carried out in the absence of theophylline in order to determine whether this substance has an inhibitory effect on adenylate cyclase, stimulated by 10 mM NaF. To minimize hydrolysis of the reaction product by phosphodiesterase excess unlabeled cyclic AMP (0.5 mg/ml, corresponding to approximately 1.5 mM) is added. The activities in these experiments are low and vary considerably, and otherwise non-observable fluorescent spots appear on the thin-layer plate, presumably due to the action of phosphodiesterase and other phosphatase activities. Since no increase in adenylate

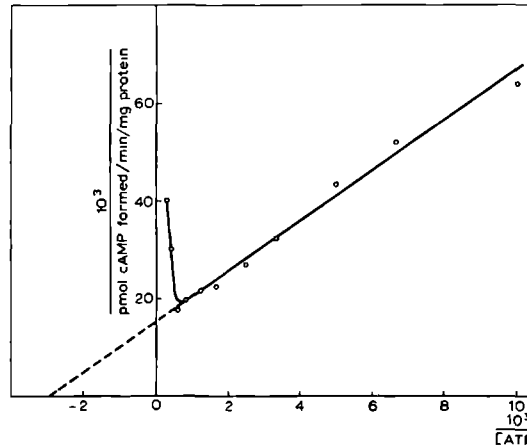

Fig. 8. Lineweaver-Burk plot for the relation between fluoride stimulated adenylate cyclase activity and substrate concentration. Freshly prepared enzyme preparation is incubated for 15 min with varying ATP concentrations in the presence of 10 mM NaF.

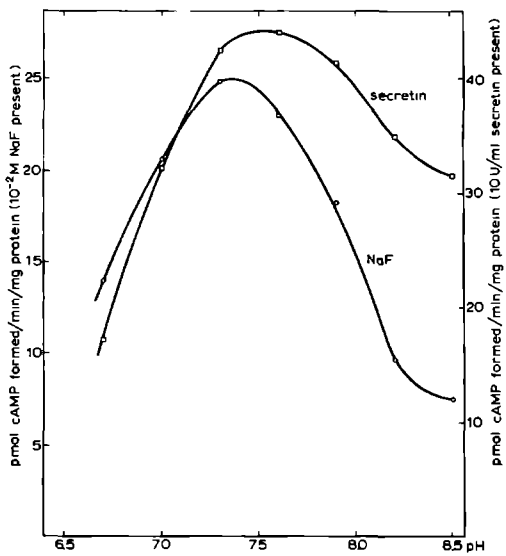

Fig. 9. Effect of pH on fluoride stimulated (o——o) and secretin stimulated (¤——¤) adenylate cyclase activity. Freshly prepared enzyme preparations are incubated in media prepared with 30 mM TRIS/30 mM HEPES buffers. The incubation time is 15 min in the presence of NaF and 10 min in the presence of synthetic secretin.

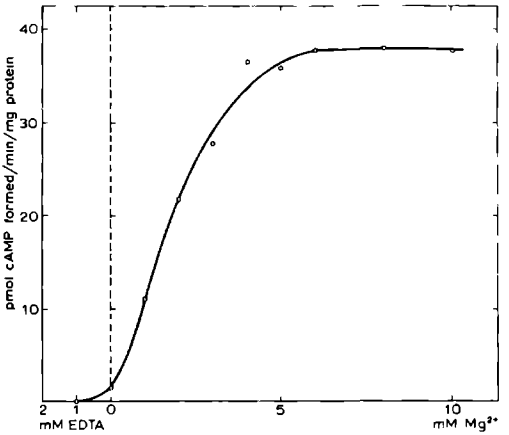

Fig. 10. Effects of magnesium ion and EDTA on fluoride stimulated adenylate cyclase. Freshly prepared enzyme preparation is incubated in the presence of varying $MgCl_2$ concentrations or of 1 mM EDTA. These concentrations are exclusive of 0.5 mM Mg^{2+} and EDTA deriving from their presence in the enzyme preparation.

cyclase activity is found, theophylline has been included in all other assays to inhibit breakdown of cyclic AMP.

Trypsin-inhibitor is added to the assay medium in order to prevent action of trypsin from the zymogen granules on the enzyme. An effect of trypsin on hormone stimulated adenylate cyclase in fat cells has been reported (Rodbell et al., 1970). In control experiments the presence of this inhibitor has no adverse influence on rat brain adenylate cyclase activity. Therefore, even though omission of trypsin inhibitor in some pancreatic adenylate cyclase assays shows no clear decrease in enzyme activity, it has been added routinely to the assay medium.

Effect of secretin and pancreozymin on adenylate cyclase activity

Rat pancreatic adenylate cyclase, in the absence of NaF, is stimulated by the hormone secretin (Fig. 11). Secretin in the final concentrations of 0.01 to 10 U/ml causes a progressive increase in activity, which remains constant at still higher concentrations. The maximal activity is slightly less than the maximal NaF-stimulated activity. The pH-optimum for the secretin-stimulated activity is virtually the same as for the NaF-stimulated activity, although the curve for secretin decreases less steeply on the alkaline side (Fig.9). The secretin (10 U/ml)-stimulated enzyme rate is constant for at least 15 minutes of incubation and up to 150 µg enzyme protein per tube (Fig. 12). The effect of secretin on the adenylate

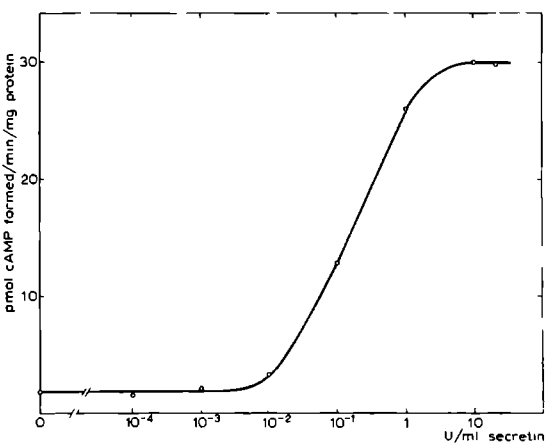

Fig. 11. Effect of synthetic secretin on adenylate cyclase activity. Freshly prepared enzyme preparation is incubated for 10 min in the presence of varying concentrations of synthetic secretin.

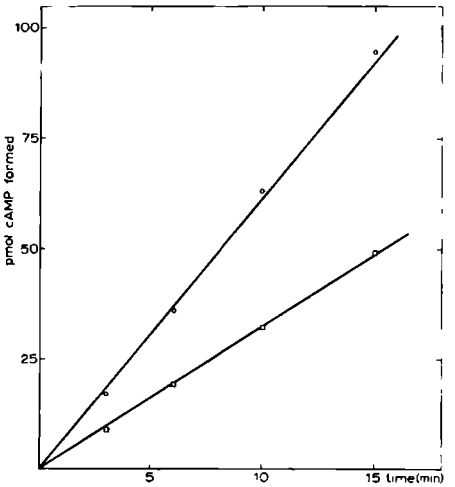

Fig. 12. Linearity of secretin stimulated adenylate cyclase assay. Two dilutions of freshly prepared enzyme preparations are incubated for varying lengths of time in the presence of 10 U/ml synthetic secretin (o———o: 167 µg protein; □———□: 84 µg protein).

cyclase activity is not additive to that of 10 mM NaF, as is commonly observed for the effects of hormone and fluoride on this enzyme in other tissues. Half maximal activation occurs at a secretin concentration of 0.15 U/ml. Since the secretin preparation has an activity of 3 U/µg and the M.W. is 3056, this concentration is equivalent to 1.5×10^{-8}M.

The enzyme, in the absence of NaF, is also stimulated by pancreozymin (Fig.13). A similar curve as with secretin is obtained for highly purified pancreozymin in concentrations up to 150 U/ml. Although higher contrations could not

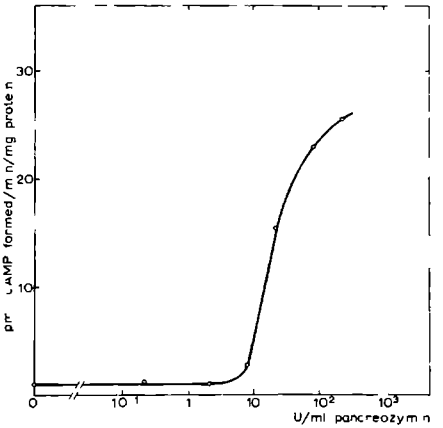

Fig. 13. Effect of highly purified pancreozymin on adenylate cyclase activity. Freshly prepared enzyme preparation is incubated for 10 min in the presence of varying concentrations of purified pancreozymin.

be tested due to the limited amount of material available, the activity seems to reach a maximum at about the same level as for secretin, and slightly less than the maximal NaF stimulated activity. The effect of pancreozymin is, like that of secretin, not additive to that of 10 mM NaF. Half maximal activation occurs at a pancreozymin concentration of approximately 20 U/ml. Since the pancreozymin preparation has an activity of 3 U/µg and the M.W. is 3915, this concentration is equivalent to 1.5×10^{-6}M. This half maximally activating concentration is 100 times higher than that for secretin. The enzyme activity in the presence of a maximally activating concentration of either hormone is not further increased by addition of the other hormone,

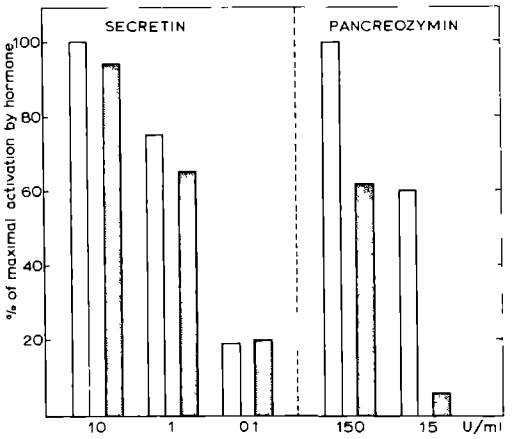

Fig. 14. Effect of oxidation of synthetic secretin and purified pancreozymin on their activation of adenylate cyclase. To solutions of 10 U synthetic secretin and 150 U highly purified pancreozymin in 50 µl of a solution of 0.6% hydrogen peroxide in 1 M acetic acid is added. After 45 min at room temperature each solution is diluted with 500 µl distilled water and then lyophilized. Freshly prepared enzyme preparation is incubated for 10 min in the presence of different concentrations of the oxidized and non-oxidized hormones.
Other conditions are as described in section 3.2. The results are expressed as percentage of the maximal obtainable activation by the relevant hormone.
Open bars: untreated hormones.
Shaded bars: oxidized hormones.

i.e. there is no additive effect of the two hormones.

It occurred to us that the high half-maximal activating concentration of pancreozymin and the non-additivity of the hormone effects might mean that the purified pancreozymin preparation is contaminated by a small amount of secretin. This possibility has been tested by utilizing the different effects of oxidation on the two hormones. Small amounts of each hormone are subjected to oxidation by hydrogen peroxide according to Mutt (1964) before incubation. This treatment destroys 90% of the physiological effects of pancreozymin in the rat, on enzyme secretion (Mutt, 1964) as well as on fluid secretion (Heatly, 1968), while secretin is not affected by it. The effect of prior oxidation of the hormones on their activation of rat pancreatic adenylate cyclase is shown in Fig. 14. Both maximally and submaximally stimulating concentrations are used. The activation caused by secretin (0.1, 1 and 10 U/ml) is virtually unaffected at any concentration. Pancreozymin in submaximally activating (15 U/ml) and nearly maximally activating concentration (150 U/ml) demonstrates 90% and 40% losses in activation after oxidation. Both losses represent approximately 90% inactivation of the hormone by oxidation, as can be seen from the activating effect of 15 U/ml non-oxidized pancreozymin shown in Fig. 14.

Since the purified hormone preparations were available only in limited amounts, clinical preparations have also been tested. Secretin preparations from two sources stimulate pancreatic adenylate cyclase, but the maximally obtainable activity is much lower than in the case of the pure preparation. Furthermore, after reaching a maximum with increasing amounts of the hormone, the activity falls

again to a lower level at still higher concentrations of secretin (4 U/ml, Sigma). Addition of 4 U/ml of this secretin preparation to an assay medium containing 10 mM NaF lowers the NaF stimulated enzyme activity by 90% (Fig. 15). These effects are not observed with the synthetic preparation, suggesting that the clinical preparation contains an inhibitor of adenylate cyclase.. With pancreozymin (Sigma) no enzyme activation is observed, while with pancreozymin (Boots) a similar behaviour as with secretin (Sigma) is observed except that the fluoride activation is not decreased.

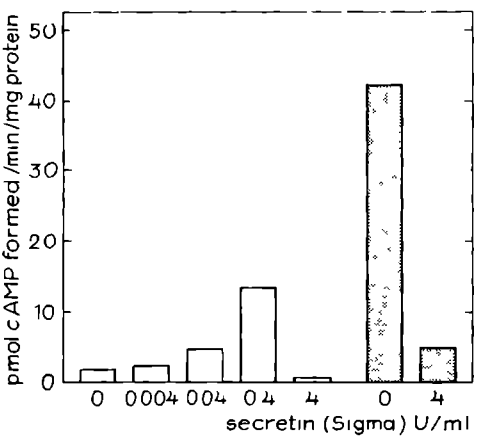

Fig. 15. Effect of a commercial secretin preparation on basal and fluoride stimulated activity.
Freshly prepared enzyme preparation is incubated with varying concentrations of secretin (Sigma). In some incubations 10 mM NaF is also present.

Light bars: no NaF present

Dark bars: 10 mM NaF present

Effects of other hormones and autonomic stimulants

In view of the structural similarity of secretin and glucagon on the one hand, and pancreozymin and gastrin on the other, the effects of preparations of glucagon and gastrin on the enzyme have also been tested. With glucagon no activation is found, which is also true for pentagastrin, a synthetic, partial peptide of gastrin.

Since pancreatic enzyme output is known to be stimulated by acetylcholine and carbachol, these compounds have also been tested. Neither compound, in concentrations ranging from 10^{-6} to 10^{-2}M, causes accumulation of cyclic AMP above the basal level.

The same observation is made when adrenalin, noradrenalin or isoprenaline are tested in this concentration range. No increase of the basal activity is obtained. Neither does adrenalin change the activation of the enzyme caused by 10 U/ml secretin.

Effects of calcium ions

Inhibition of adenylate cyclase activity by calcium ion has been reported for several tissues. Therefore, the effect of calcium on the pancreatic enzyme preparation has also been investigated. Calcium, added in concentrations ranging from 0.1 to 5 mM progressively inhibits the enzyme activity in the presence of either secretin or fluoride (Fig. 16).

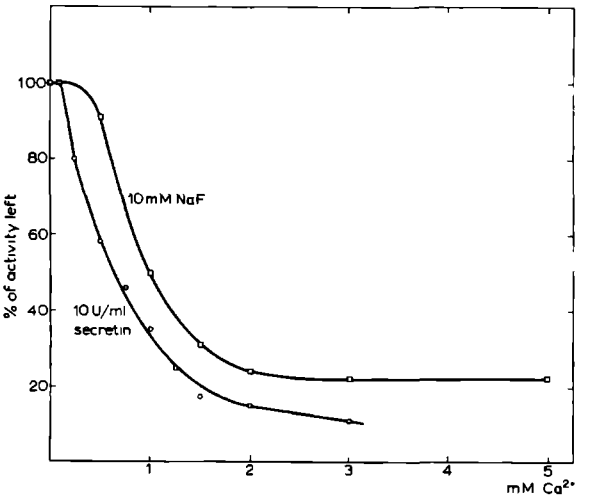

Fig. 16. Inhibition by calcium ion of fluoride stimulated and secretin stimulated adenylate cyclase activity.
Freshly prepared enzyme preparation is incubated with varying calcium concentrations in the presence of 10 mM NaF for 15 min or in the presence of 10 U/ml synthetic secretin for 10 min.
Adenylate cyclase activity is plotted as percentage of activity in the absence of calcium.

Changes in adenylate cyclase activity after pancreatic stimulation in vivo

The large variability in both basal and NaF- or secretin-stimulated adenylate cyclase activities between individual animals (Table VI, column I) has led us to measure the enzyme activity in starved rats (column II) and in rats maximally stimulated in vivo by feeding and injection of secretin and pancreozymin (column III).

TABLE VI

EFFECT OF EXTENSIVE STIMULATION IN VIVO ON ADENYLATE CYCLASE ACTIVITY

One group of 4 rats is starved for 20 hours prior to removal of the pancreas (col.II). Another group of 4 rats has free access to food and is intravenously injected with 5 U each of pancreozymin (Boots) and secretin (Boots) 17 hrs and with 10 U of each hormone 1½ hrs before sacrifice (col.III). Enzyme preparations from each animal are made and assays are carried out in the presence of 10 mM fluoride, 10 U/ml secretin or no addition (10 min incubation).
Other conditions are as described in 3.2.
Enzyme activity is given in pmol cAMP formed/min/mg protein.
Column I shows for purposes of comparison the range for the specific activity in all animals investigated previously.
In columns II and III averages are given with S.E. of the mean.

Addition	I Normal range	II Starved, no hormones	III Food, hormones injected	Difference between II and III
None	0-3	1.1±0.4	1.9±0.8	N.S.
10 mM NaF	±15-40	19.2±1.8	45.0±4.8	P<0.001
10 U/ml secretin	±15-40	16.3±1.6	35.4±2.4	P<0.001

While the basal activity is not significantly increased, the NaF- and secretin-stimulated activities are significantly increased in the latter group of animals. Since the activities are expressed per mg protein present in the preparation, the increased activities could be due to a lowering of the protein content through secretion of the digestive enzymes. This explanation is unlikely for the following reasons. The preparation of the particulate adenylate cyclase involves hypotonic treatment in order to lyse the zymogen granules and to remove soluble proteins. The slightly higher protein yield, which we find in the preparations from starved animals can account for only about one third of the difference of the specific activities in columns II and III.

3.4. DISCUSSION

The basal activity of rat pancreatic adenylate cyclase (0-3 pmol cyclic AMP formed/min/mg protein) is lower than that reported for most other tissues. Addition of fluoride raises the activity to a level (15-40 pmol cyclic AMP formed/min/mg protein) which is more commonly found in other tissues. The properties of the NaF-stimulated enzyme are rather similar to those observed for the enzyme in several other tissues, e.g. the pH optimum of 7.4, the K_m value of 0.3 mM for ATP, the Mg^{2+} activation and the Ca^{2+} inhibition. The substrate inhibition (Fig.8) is not uncommon for the fluoride stimulated enzyme. Pohl et al. (1971) reported a similar substrate inhibition in rat liver plasma membranes, when the adenylate cyclase was stimulated by NaF, but with glucagon as the activating agent there was no substrate inhibition. Although these authors did not

calculate the K_m value for ATP, their v-[S] curves suggest that it is the same in the presence of either activating agent.

The most interesting effects are of course those of various hormones on the enzyme activity. First, the synthetic secretin preparation used in our experiments has a strongly activating effect. This observation could support the conclusion of Case and Scratcherd (1972) that secretin would exert its action on water and electrolyte secretion via cyclic AMP. It would also agree with the observation (Case et al., 1972) that cyclic AMP levels rise in the isolated cat pancreas after perfusion of secretin as well as in the pancreas of the anesthetised cat after infusion of this hormone. To our knowledge, a stimulating effect of secretin on adenylate cyclase has so far only been reported for one other tissue, namely fat cells of the rat (Rodbell et al., 1970).

Pancreatic adenylate cyclase is not stimulated by glucagon, although there is a great similarity in structure between glucagon and secretin, which are thought to have been derived from the same parent structure during evolution (Mutt and Jorpes, 1967). Rat liver adenylate cyclase, on the other hand, responds to glucagon, but not to secretin (Rodbell et al., 1970). This supports once again the theory that adenylate cyclase in different tissues can have highly specific hormone receptor sites. The receptor site in fat cells appears to be less specific, since their adenylate cyclase is stimulated by secretin as well as by glucagon, although the maximal activation by the latter hormone is lower than that by the former (Rodbell et al., 1970). Trypsin treatment of the fat cells, moreover, completely

abolishes the activation by glucagon, but only partially that by secretin, suggesting different receptor sites for the two hormones. A suggestion for a competition between glucagon and secretin for the pancreatic adenylate cyclase receptor site is given by the observation of flow inhibition upon glucagon administration to dogs receiving continuous intravenous infusion of secretin (Dijck et al., 1969).

One further comment about the absence of pancreatic adenylate cyclase stimulation by glucagon is in order. The hormone is thought to exert its insulin-releasing action in pancreatic β-cells through cyclic AMP (Levine, 1970) and would thus be expected to stimulate the adenylate cyclase present in these cells. Since however, the islets of Langerhans form only a few percent of the total amount of pancreatic tissue, they will probably have only a minor part of the total pancreatic adenylate cyclase activity. Hence, a stimulation of this small fraction of enzyme activity by glucagon might well go unnoticed in our experiments.

The effects on pancreatic adenylate cyclase of compounds affecting enzyme secretion have also been investigated in some detail. Pancreozymin causes nearly the same maximal stimulation of rat pancreatic adenylate cyclase as secretin, though the half-maximal stimulating concentration is 100 times as high. The relative insensitivity for pancreozymin could mean that this effect is due to contamination of the pancreozymin preparation with secretin. However, the results of the experiments, in which the hormones were subjected to prior oxidative treatment, appear to rule out this possibility. The non-additivity of the effects of maximally stimulating concentrations of pan-

creozymin and secretin suggests that both hormones exert their action on the same adenylate cyclase.

The question arises which adenylate cyclase this would be: the adenylate cyclase of the enzyme secreting cells, or that of the fluid secreting cells. The possibility that both types of cells would have the same adenylate cyclase is highly improbable, because of the clearly different physiological actions of secretin and pancreozymin. A more likely possibility is that the adenylate cyclase system in the fluid secreting cells responds to both hormones. This hypothesis is supported by the observation of Heatley (1968) that pure secretin in the pancreas has no effect on enzyme secretion, while pure pancreozymin stimulates both enzyme secretion and fluid secretion. The fact that in his experiments the pancreozymin on a molar basis is less active than secretin on fluid secretion may explain, at least in part, why in our experiments much more pancreozymin than secretin is needed for the same effect on adenylate cyclase. Pentagastrin, which resembles pancreozymin and gastrin in having an identical C-terminal tetrapeptide, and gastrin stimulate pancreatic enzyme secretion in vivo (Morley et al., 1965; Grossman, 1967; Wormsley et al., 1966) as well as in vitro (Kulka and Sternlicht, 1968). However, pentagastrin has no stimulating effect on rat pancreatic adenylate cyclase.

The other possibility is that the adenylate cyclase activity represents the enzyme present in the enzyme secreting cells. An argument favoring this assumption is that in the rat these cells represent the major part of total pancreatic mass (Hegre et al., 1972). The repeated reports supporting a role of cyclic AMP in enzyme

secretion (see section 2.4.) are also in favor of this assumption. The same is true for the stimulation of enzyme secretion by papaverine, in the face of a lowering of fluid secretion (see section 2.3.). However, there are reports denying such a role and our own experiments with exogenous cyclic AMP could not confirm the earlier findings of Ridderstap and Bonting (1969c). Experiments in which cyclic AMP levels were measured in living pancreas cells before and after stimulation also yield conflicting evidence. Benz et al. (1972) did not find elevation of these levels in guinea pig pancreas slices in response to pancreozymin or cholinergics. The opposite observation was made by Case et al. (1972), who observed elevation of cyclic AMP levels in cat pancreas in vivo and in vitro after pancreozymin as well as after acetylcholine stimulation. However, these authors do not consider this as evidence for an intermediate function of cyclic AMP, firstly because enzyme secretion and levels of cyclic AMP change on a different time basis and secondly because atropine blocks the stimulation of enzyme secretion but not the increase in cyclic AMP levels after stimulation.

Although cholinergic agents are known to stimulate enzyme secretion in vivo as well as in vitro (Schramm, 1967), the absence of a stimulating effect of acetylcholine and carbachol on the adenylate cyclase activity in our experiments is not in conflict with this finding, since these substances generally do not affect adenylate cyclase activity (Murad et al., 1962).

The foregoing considerations make it obvious, that an unequivocal localization of the adenylate cyclase activities within the pancreas is very much needed.

The absence of a stimulating effect of adrenergic compounds on pancreatic adenylate cyclase is in agreement with the finding that these compounds do not elicit any stimulatory response in the pancreas (Schramm, 1967). The fact that adrenalin does not inhibit secretin stimulated adenylate cyclase is of interest, because in the isolated rabbit pancreas alpha-adrenergic stimulation has been observed to inhibit fluid secretion (Hubel, 1970).

We may be able to offer an explanation for the large variability in both basal and fluoride-or secretin-stimulated adenylate cyclase activities in normal rats. The activities in enzyme preparations from starved animals are at the lower end of the range, while preparations from the animals stimulated by free access to food and double injections of secretin and pancreozymin have an activity coinciding with the upper end of the normal range. This suggests that the large range of activities in the normal rats is due to differences in food uptake immediately prior to sacrifice of the animals.

CHAPTER IV

IMPROVED METHOD FOR THE ASSAY OF ADENOSINE 3',5' MONOPHOSPHATE PHOSPHODIESTERASE IN TISSUE HOMOGENATES

4.1. INTRODUCTION

In order that 3',5'-AMP may act as a hormone messenger, it is essential to have an enzyme, which will remove 3',5'-AMP after it has been formed through hormone stimulated activity of adenylate cyclase and has initiated the intracellular process required for expression of the hormone message. Hence, it is necessary to investigate the presence and the properties of the phosphodiesterase enzyme in the course of a study of the possible role of 3',5'-AMP in exocrine pancreatic secretion.

A survey has been made of the various assay methods that have been reported for this enzyme. Having selected the method of Loten and Sneyd (1970) it has been found that this method can give erroneous results when crude enzyme preparations are used. This has necessitated a revision of this method, which is described in this chapter.

The 3',5'-AMP phosphodiesterase activity can be determined in various ways. First, the residual amount of 3',5'-AMP after incubation can be measured (Pöch, 1971). Secondly, the amount of 5'-AMP, the product of the enzymatic reaction, can be determined by various methods (Schultz et al., 1966; Weiss et al., 1972). In the third approach this product is further hydrolysed by means of an added excess of 5'-nucleotidase to adenosine and inorganic

phosphate, the latter product being determined (Butcher and Sutherland, 1962; Nair, 1966). Finally, 3',5'-[^{3}H]-AMP can be used as substrate and the amount of [^{3}H] -adenosine formed is determined. This can be done in two ways. In the first method, the reaction mixture is added to a suspension of anionic exchange resin, whereupon the amount of [^{3}H]-adenosine is determined as the total amount of radioactivity of the whole suspension, the radioactivity from unchanged 3',5'-[^{3}H] -AMP being quenched by the resin (Thompson and Appleman, 1971a). In the second method, the reaction mixture is applied to a column of the same resin and [^{3}H] -adenosine is eluted with water (Loten and Sneyd, 1970).

All these methods have disadvantages. The method of Pöch (1971) is liable to yield relatively large errors at low conversion rates of the substrate, since it requires measuring small differences. In the methods of Butcher and Sutherland (1962) and Nair (1966) the measurement of P_i after hydrolysis of the resulting 5'-AMP is not sensitive enough for very low substrate concentrations, such as are necessary to determine the 3',5'-AMP phosphodiesterase activity with low K_m value (Thompson and Appleman, 1971a; Loten and Sneyd, 1970; Huang and Kemp, 1971; Klotz et al., 1972; Ashcroft et al., 1972; Jard and Bernard, 1970). The

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In this chapter and in chapter V the abbreviation 3',5'-AMP instead of cyclic AMP will be used for the compound adenosine 3',5'monophosphate in order to prevent confusion with other types of compounds. Other abbreviations are: 5'-AMP: adenosine 5'monophosphate; 5'-IMP: inosine 5'monophosphate; P_i: inorganic phosphate.

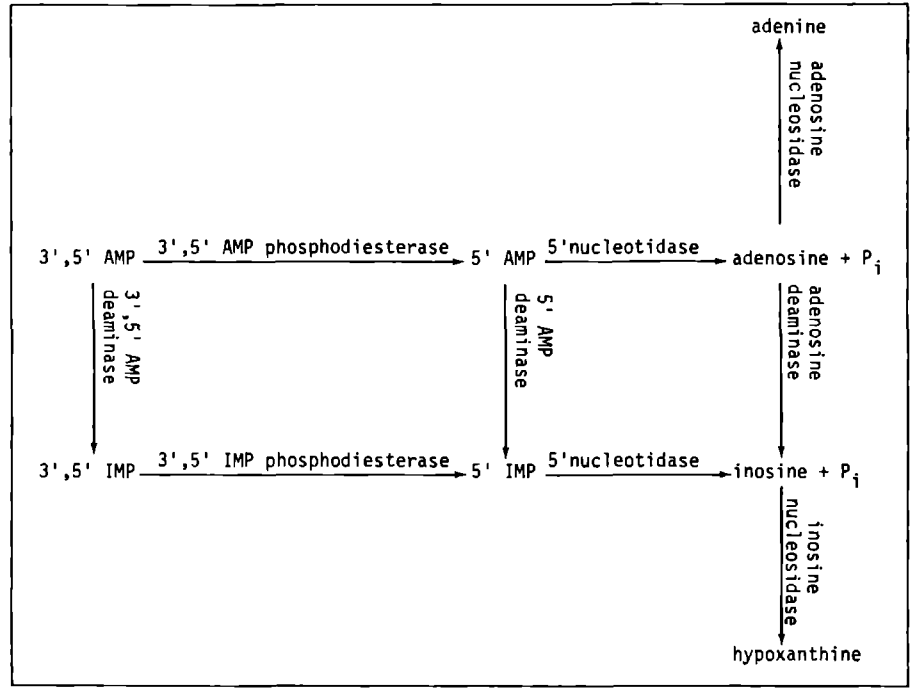

Fig. 17. Pathways of 3',5'-AMP conversion upon incubation with tissue homogenates.

measurement of [^{3}H] - adenosine production is very sensitive, but cannot be applied to homogenates or other unpurified enzyme preparations. These preparations usually contain enzymes converting 5'-AMP to products other than adenosine (Therriault and Winters, 1970; Gulyassy and Oken, 1971), and these products stay on the resin, thus leading to erroneous results for 3',5'-AMP hydrolysis. The approach of Schultz et al. (1966) and Weiss et al. (1972), in which the production of 5'-AMP is measured, is even more sensitive to errors caused by enzymatic conversion of 5'-AMP.

In this chapter a modification of the method of Loten and Sneyd (1970) is described, in which not only adenosine, but also inosine, adenine and hypoxanthine are eluted from a column of anionic exchange resin, since these compounds can also be reaction products through the reactions presented in Fig. 17. The sum of all radioactive breakdown products, which is equal to the amount of 3',5'-AMP converted by phosphodiesterase, is then measured.

4.2. METHODS

Determination of 3',5'-AMP phosphodiesterase activity

Whole homogenate of rat pancreas in 0.9% NaCl is used as the enzyme preparation. Unless otherwise specified, the incubation medium contains 66 mM TRIS-HCl (pH 8.5), 1mM $MgCl_2$, either 2 mM or 4 µM 3',5'-[^{3}H]- AMP (about 0.2 µCi) and 25 µl homogenate in a total volume of 125 µl. After addition of 25 µl of a solution of 100 µg 5'-nucleotidase in 1 ml of 50 mM $MgCl_2$, the mixture is incubated for 30 min at 37°C, whereupon the mixture is centrifuged and the supernatant is used for determination of the radioactive reaction

products. It can also be stored overnight at -20°C before centrifugation and further treatment.

The procedure finally adopted for the determination of the reaction products is as follows. A 100 µl aliquot of the supernatant, obtained after 5'-nucleotidase treatment, is applied to a 15mm long column of Dowex AG 1 - X2 resin in a Pasteur pipette (diameter 7 mm). The column is eluted with 10 ml 0.1 M $NaHCO_3$. The total eluate is mixed with 10 ml Insta-Gel scintillation fluid and the radioactivity is counted in a liquid scintillation spectrometer (Packard Tri-Carb, model 3380).

Separation of nucleosides and nucleotides

Nucleosides and nucleotides are separated by thin-layer chromatography on Chromar-Sheet 500 according to Woods and Waitzman (1970). The thin layers are developed with a mixture of 2-propanol, ethylacetate and 13 M ammonia (59 : 29: 16, by vol., system I). This system does not permit separation of 5'-AMP and 5'-IMP. Separation of these compounds is performed with a mixture of 2-propanol, ethylacetate and 8.5 M ammonia (30 : 44.5 : 25.5, by vol., system II). After development of the thin layer, the spots are visualized under 254 nm ultraviolet light, cut out and transfered to 5 ml 0.5 M NH_4OH in a scintillation vial. After 1 hour the mixture is neutralyzed with 3 N HCl and 10 ml of Insta-Gel is added, whereupon radioactivity is counted.

4.3. RESULTS AND DISCUSSION

Estimation of phosphodiesterase activity by measuring [^{3}H]-adenosine production according to Thompson and

Appleman (1971a) or Loten and Sneyd (1970) yields very low results, when these methods are applied to rat pancreatic homogenates. Hence, the identity of the labelled products, present before and after the second incubation step, has been determined by thin-layer chromatography (system I). The results of these experiments, both at low and high substrate concentrations are shown in Fig. 18. In both cases

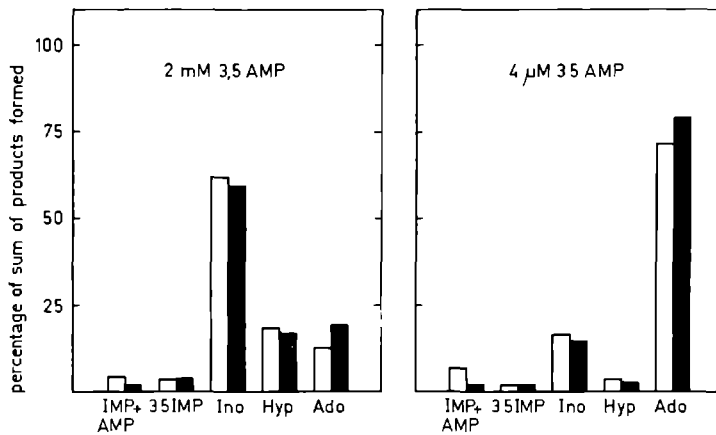

Fig. 18. Relative amounts of radioactive products formed from 3',5'-[^{3}H]-AMP before (open bars) and after (closed bars) incubation with 5'-nucleotidase. Separation of the products is performed by thin-layer chromatography as described in 4.2. Results are expressed as percentages of the total radioactivy recovered from the thin-layers. Recovery of radioactivity, applied to the thin-layers, is in all cases better than 95%. IMP = 5'-IMP, AMP = 5'-AMP, Ino = inosine, Hyp = hypoxanthine, Ado = adenosine.

even before addition of 5'-nucleotidase the major part of the resulting 5'-AMP has already been converted, which is probably due to the presence of a high 5'-nucleotidase activity in rat pancreas (Putzke and Ewinst, 1971). The second incubation step leads to nearly complete conversion of 5'-AMP to adenosine. When the high substrate concentration (2 mM) is used, inosine is the most important breakdown product, followed by adenosine. This has also been found in rat heart supernatant (Therriault and Winters, 1970). At low substrate concentration (4 µM) the situation is reversed, which may be explained by a relatively high K_m value for adenosine of the enzyme adenosine deaminase, such as found in calf intestinal mucosa (Km = 35 µM; Coddington, 1965). Hypoxanthine is another breakdown product present in appreciable amounts, especially at high substrate concentration. This is probably due to its formation from inosine by the action of inosine nucleosidase. Adenine, which would be formed by adenosine nucleosidase from adenosine, is only found in trace amounts (± 0.5% of the 3',5'-AMP converted), when the high substrate concentration is used.

A hitherto unreported observation is the formation of a small amount of 3',5'-IMP. This may be due to the action of a specific 3',5'-AMP deaminase or possibly to the less specific action of 5'-AMP deaminase. With the aid of the developing system II it has been shown that the amount of 5'-IMP is no more than about 10 - 20% of the sum of both compounds at either substrate concentration.

The pathways by which 3',5'-AMP can be broken down are shown in Fig. 17. The question now arises whether 3', 5'-IMP is an important intermediate in the breakdown of

3',5'-AMP. This can be only the case if 3',5'-IMP is rapidly converted via 5'-IMP into inosine. This is improbable for the following reasons. First, at low substrate concentrations the main breakdown product is adenosine, which cannot be formed via 3',5'-IMP. Secondly, upon shorter incubation times at high substrate concentration the larger amount of adenosine formed and a smaller amount of inosine suggest that the hydrolytic pathway via 5'-AMP and adenosine is the more dominant one. Finally, upon inhibition of 3',5'-AMP phosphodiesterase by addition of 10 mM theophylline or 0.1 mM papaverine no increase in the amount of 3',5'-IMP is found.

Returning to the method of Thompson and Appleman (1971a) we can show that this method is unsuitable since the reaction products remain adsorbed to the resin in different proportions. This has been tested by mixing 500 µl 0.37 mM adenosine or inosine solution in 66 mM TRIS-HCl (pH 8.5) with 500 µl of a 1:4 aqueous suspension of the exchange resin. Measurement of the optical density at 256 nm of the supernatant after centrifugation reveals that 30 - 40% of the adenosine and 80% of the inosine have been adsorbed on the resin. This leads to erroneously low values for [^{3}H]-adenosine, thus making this method unsuitable for phosphodiesterase assays in pancreas homogenates.

Loten and Sneyd (1970) apply the reaction mixture to a column of the same resin and elute this column with water. However, we have observed that moderate volumes of water elute only adenosine, while inosine, adenine and hypoxanthine are retained on the column. Therefore, we have tested as eluent solutions of various electrolytes at different ionic strenghts. Best results are obtained with 10 ml 0.1 M $NaHCO_3$, as shown in Fig. 19. This figure shows

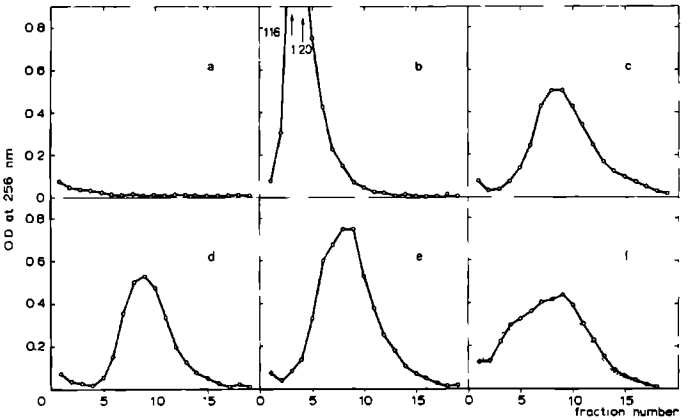

Fig. 19. Elution patterns of 3',5'-AMP (10.1 mM; a), adenosine (0.37 mM; b), inosine (0.37 mM; c), hypoxanthine (0.73 mM; d), adenine (0.74 mM; e) and a mixture of equal volumes of these 5 solutions (f) from Bio-Rad AG 1 -X 2 200-400 mesh, chloride form. Length of the columns in Pasteur pipettes (diameter 7 mm) is 15 mm; a 0.5 ml aliquot of solutions of the compounds in 66 mM TRIS -HCl (pH 8.5) is placed on the top of the column, which is then eluted with 18 x 0.5 ml 0.1 M $NaHCO_3$. The absorbance of the eluted fractions is measured at 256 nm. Absorbance pattern of the TRIS - buffer virtually coincides with pattern a. The calculated absorbance pattern for the mixture is indicated by the dotted line in f.

the elution patterns of the tested compounds, alone and in combination, in the total eluate. It is clear that, while 3',5'-AMP is completely retained, the other substances are virtually completely eluted. Other nucleotides, 3',5'-IMP, 5'-AMP and 5'-IMP, are retained like 3',5'-AMP.

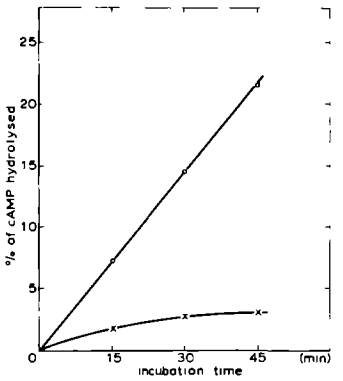

Fig. 20. Time course of 3',5'-AMP hydrolysis by rat pancreas homogenate. The upper line (o———o) is obtained with the method described in 4.2.; column elution with 10 ml 0.1 M $NaHCO_3$. The lower curve (x———x) is obtained by elution with 10 ml of water. Initial 3',5'-AMP concentration 2 mM.

In Fig. 20 our method with $NaHCO_3$ elution is compared with that of Loten and Sneyd (1970) involving elution with water. It is obvious that measurement of adenosine production by elution with water gives - at least for rat pancreas - erroneously low and non-linear results. Elution with 0.1 M $NaHCO_3$, on the other hand, yields a linear relationship between 3',5'-AMP conversion and time. The results obtained by this procedure are quite reproducible (duplicate agreement expressed as coefficient of variation: 3%) and they agree within the experimental error with the results obtained by thin-layer chromatography. Our method is suitable for the assay of 3',5'-AMP phosphodiesterase in tissue homogenates and crude enzyme preparations at

micromolar to millimolar substrate concentrations. Its use is recommended for all tissues with an appreciable deaminase activity. Chapter V describes detailed results of studies on the 3',5'-AMP phosphodiesterase activity in rat pancreas.

CHAPTER V

PRESENCE AND PROPERTIES OF ADENOSINE 3',5'MONOPHOSPHATE PHOSPHODIESTERASE IN RAT PANCREAS

5.1. INTRODUCTION

In the previous chapter a reliable method for measurement of 3',5'-AMP phosphodiesterase has been described. This method is now used to determine the presence and properties of this enzyme in rat pancreas. Since in several other tissues the simultaneous occurrence of phosphodiesterase activities with low and high K_m has been described (Thompson and Appleman, 1971a, 1971b; Loten and Sneyd, 1970; Huang and Kemp, 1971; Klotz et al., 1972; Ashcroft et al., 1972; Jard and Bernard, 1970; Franks and MacManus, 1971), special attention is paid to kinetic aspects. It will be shown that phosphodiesterase activity in rat pancreas behaves kinetically as if consisting of two distinct activities, one with high and one with low substrate affinity. The physiological significance of this finding will be discussed.

As already stated, processes which are mediated by 3',5'-AMP can be influenced by compounds, which modify the activity of 3',5'-AMP phosphodiesterase. The methylxanthines theophylline and caffeine and various other compounds, such as papaverine, are well known inhibitors of this enzyme. Since we have used these compounds as tools in studying the role of 3',5'-AMP in enzyme secretion by the isolated rabbit pancreas (Chapter II), their effects on phosphodiesterase activity have also been examined. It will be shown that the

observed effects are compatible with the conclusion that the effects of these compounds in the isolated organ are due to the inhibition of phosphodiesterase activity.

Various other properties of the enzyme, including the effects of other compounds affecting exocrine secretion, such as pancreozymin, secretin and carbachol, are also described.

5.2. METHODS

Tissue preparation and 3',5'-AMP phosphodiesterase assay

Two-three month old Wistar rats with free acess to food and water are sacrificed and the pancreas is removed. After removal of fat and connective tissue, the tissue is minced and homogenized in 4 vol. 0.9% NaCl in a Potter-Elvjehem homogenizer. The homogenate is then filtered through four layers of medical gauze and stored at -20°C for up to a week.

3',5'-AMP phosphodiesterase is measured according to the modified method of Loten and Sneyd (1970), which is described in section 4.2. Activity is expressed as nmol 3',5'-AMP converted per min per mg protein. Protein is measured as described in section 2.2.

Calculations

For two separate enyzme activites I ($V_{max,1}$, $K_{m,1}$) and II ($V_{max,2}$, $K_{m,2}$), which convert the same substrate, the following relation is valid at any given substrate concentration S :

$$v_{total} = v_1 + v_2 = \frac{V_{max,1} \cdot [S]}{K_{m,1} + [S]} + \frac{V_{max,2} \cdot [S]}{K_{m,2} + [S]} \qquad (1)$$

If $K_{m,1} \ll K_{m,2}$ and $V_{max,1} \leq V_{max,2}$, equation (1) can be simplified for very low values of [S] :

$$v_{total} = \frac{V_{max,1} \cdot [S]}{K_{m,1} + S} = v_1 \qquad (2)$$

and for high values of [S]:

$$v_{total} = V_{max,1} + \frac{V_{max,2} \cdot [S]}{K_{m,2} + [S]} \qquad (3)$$

5.3. RESULTS

Linearity of 3',5'-AMP phosphodiesterase assay

Incubation time and enzyme concentration must be carefully chosen to ensure linearity of 3',5'-AMP hydrolysis with respect to these parameters. At a substrate concentration of 2 mM, linearity exists up to at least 45 min and 4 mg protein per ml of incubation medium. Up to 22% substrate hydrolysis the reaction rate remains constant. At this substrate concentration incubations are therefore routinely carried out for 15 or 30 min at enzyme concentrations of 2-3 mg protein per ml of incubation medium.

At a substrate concentration of 4 µM we do not exceed incubation times of 10 min and enzyme concentrations of 0.35 mg protein per ml of incubation medium. Up to 40% of the substrate can be hydrolysed under these conditions without significant decline in reaction rate. At this substrate concentration enzyme concentrations of 0.1 mg protein per ml of incubation mixture and an incubation time of 10 min are maintained routinely; 3',5'-AMP hydrolysis under these conditions does not exceed 10%.

Effect of substrate concentration

A number of publications concerning phosphodiesterases in various tissues attributes the observed relationship between activity and substrate concentration to the occurrence of two enzymes with different affinity for 3',5'-AMP, as in rat brain (Thompson and Appleman, 1971a), rat adipose tissue (Loten and Sneyd, 1970, Klotz et al., 1972), rat kidney (Jard and Bernard, 1970), rat thymic lymphocytes, (Franks and MacManus, 1971), mouse pancreatic islets (Ashcroft et al., 1970) and rabbit skeletal muscle (Huang and Kemp, 1971). Therefore, we have made a detailed analysis of this relationship for the activity present in rat pancreas. Fig. 21 shows a Lineweaver-Burk plot for the relation between enzyme activity and substrate concentration. At low substrate concentrations a straight line is obtained, which at extrapolation yields a K_m for 3',5'-AMP of 5.8 µM and a V_{max} of 1.09 nmol 3',5'-AMP hydrolysed per min per mg protein (see equation (2) in section 5.2.). At substrate concentrations above 10 µM the curve deviates from this linear relationship, suggesting the additional presence of an activity with high K_m value. The points at high substrate concentrations can be corrected for the contribution of the low K_m activity to substrate hydrolysis (see equation (3) in section 5.2.). Plotting the corrected values in the substrate concentration range 0.1 - 2 mM in a Lineweaver - Burk plot yields a straight line, from which a K_m value for 3',5'-AMP of 282 µM and a V_{max} of 3.91 nmol 3',5'-AMP hydrolysed per min per mg protein can be calculated.

Knowing the V_{max} and K_m value of the activity with high K_m, we can also correct the activities at low substrate con-

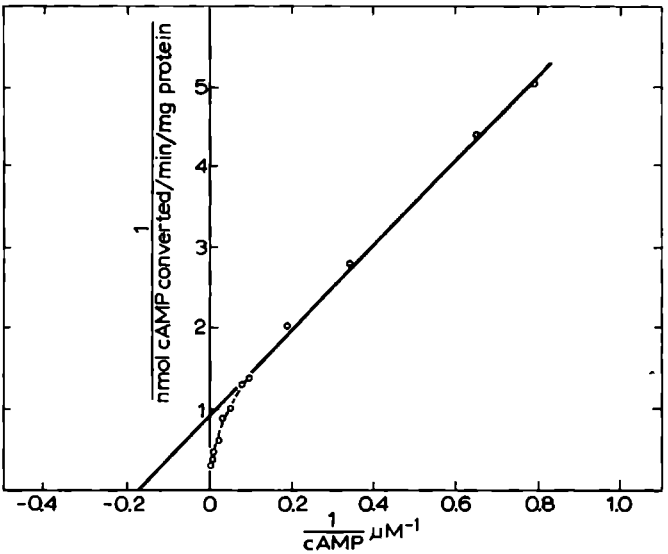

Fig. 21. Lineweaver-Burk plot for the relation of pancreatic phosphodiesterase activity and 3',5'-AMP concentration. All incubations are carried out for 10 minutes. The homogenate contains 3.24 mg protein per ml at 3',5'-AMP concentrations from 2.3 - 2085 µM and 1.65 mg protein per ml at 3',5'-AMP concentrations of 1.27 - 257 µM.

centration for the contribution of the high K_m enzyme (see equation (1) in section 5.2.). This cross-correction can be repeated in order to obtain more accurate values of the kinetic parameters. Three consecutive cross-corrections prove to be sufficient. This results in K_m values of 4.9 and 267 µM, respectively, and V_{max} values of 0.83 and 4.2 nmol 3',5'-AMP hydrolysed per min per mg protein, respectively. Figures 22a and 22b show Lineweaver-Burk plots, in which the corrected activities have been used. Subsequent experiments have all been carried out at substrate concentra-

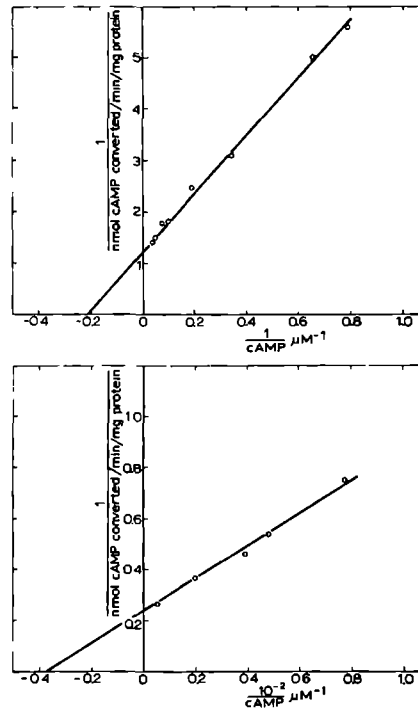

Fig. 22. Corrected Lineweaver-Burk plots at low (Fig. 22a) and high (Fig. 22b) substrate concentrations, obtained from the data in Fig. 21 after triple cross-correction as described in the text for the contributions of the high and low K_m activities.

tions of 4 µM and 2 mM, respectively. At 4 µM the activity with the high substrate affinity is mainly measured; calculation by means of the above kinetic parameters shows that at this substrate concentration the low affinity activity contributes only 14% of the observed activity. Conversely, the contribution of the low K_m activity at the high substrate

concentration of 2 mM is 16% (see equation (1) in section 5.2.).

Although the kinetic data do not definitely prove that the two activities are two different enzymes, they at least suggest that the two activites are distinct and behave independently of each other.

Influence of magnesium and calcium

The presence of magnesium is an absolute requirement for most, if not all 3',5'-AMP phosphodiesterases investigated so far, although Mn^{2+} can replace Mg^{2+} in many cases (Huang and Kemp, 1971; Cheung, 1971; Nair, 1966). In rat pancreas both activities increase sharply with rising mag-

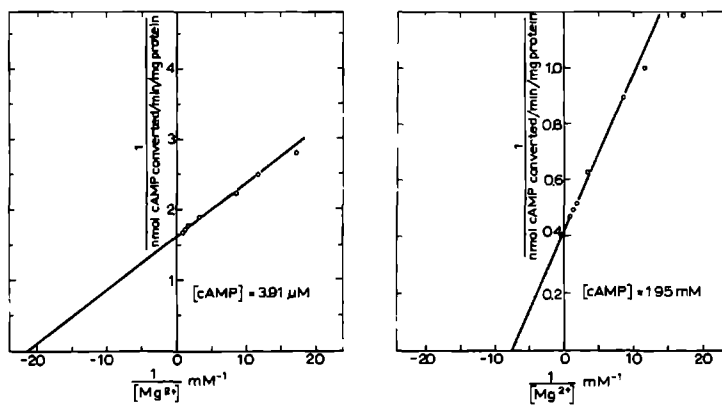

Fig. 23. Influence of magnesium on 3',5'-AMP phosphodiesterase activity at high (Fig. 23a) and low (Fig. 23b) substrate concentration, plotted in a double reciprocal way. Pancreatic homogenate is prepared in a solution of 0.2 mM EDTA in 0.9% NaCl and is then dialysed against 0.9% NaCl for 4 hours at 0°C. The diluted homogenate contains 9.6 mg protein per ml (Fig. 23a) or 0.46 mg protein per ml (Fig. 23b). Incubation times are 30 min and 10 min respectively.

nesium concentration; maximal activities are reached at approximately 0.5 mM at low substrate concentration and at approximately 0.75 mM at the high substrate concentration. If the reciprocal values of substrate hydrolysis velocity at low substrate concentrations are plotted against the reciprocal magnesium concentration, a straight line is obtained (Fig. 23a). The intercept with the axis yields the negative reciprocal value of the K_a for magnesium: this value is 48 µM. When the results at high substrate concentration are plotted in the same way, no straight line is obtained. However, after correction of the observed values for the contribution of the low K_m enzyme, a straight line is obtained (Fig. 23b), from which the K_a for Mg^{2+} for the high K_m enzyme is calculated to be 110 µM.

Since some authors report that 3',5'-AMP phosphodiesterase is at least in part dependent on low concentrations of calcium (Miki and Yoshida, 1972; Kakiuchi and Yamazaki, 1970a, 1970b; Kakiuchi et al., 1971), we have studied the effects of calcium and of EGTA, which should eliminate calcium ions present in the homogenate. Calcium in low concentrations (< 0.1 mM) has no effect on the enzyme activity, but in concentrations of 0.1 - 10 mM this cation increasingly inhibits enzyme activity, both at low and high substrate concentration (Fig. 24a and b). Addition of EGTA has no effect, except that at high concentrations (10 mM) some inhibition is seen, which may, however, be due to complexation of magnesium.

Effect of pH

The relation between activity and pH is determined at both low and high substrate concentrations. The pH-optima

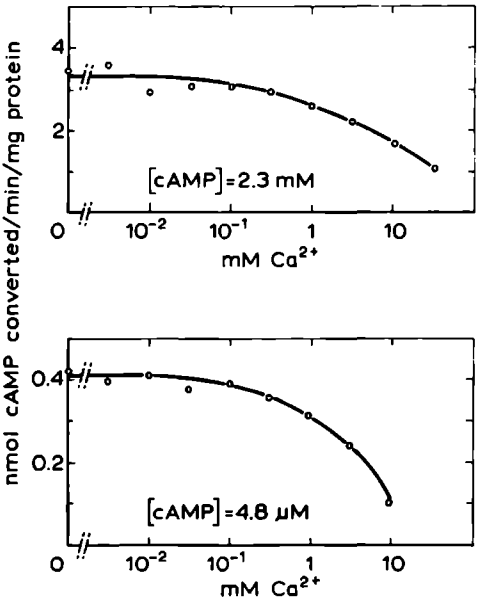

Fig. 24. Inhibition of 3',5'-AMP phosphodiesterase in rat pancreas by calcium. Freshly prepared pancreatic homogenate is dialysed during 3 periods of 1 hour against 0.9% NaCl at 0°C and diluted to a concentration of 15.8 mg protein per ml for 30 minutes incubations at high substrate concentration (Fig. 24a) or 0.74 mg protein per ml for 10 minutes incubations at low substrate concentration (Fig. 24b).

for both activities differ little from each other. In the experiments, shown in Fig. 25a and b, the optima are 8.1 and 8.2 for the high K_m and the low K_m activity, respectively.

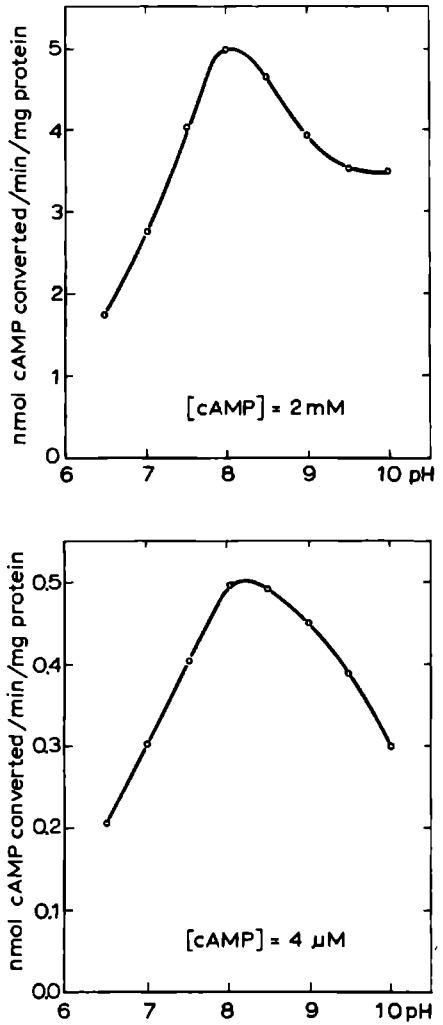

Fig. 25. Effect of pH on 3',5'-AMP phosphodiesterase activity at high (Fig. 25a) and low (Fig. 25b) substrate concentration. The pancreatic homogenate contains 17.0 mg protein per ml at high and 0.31 mg protein per ml at low substrate concentration. Incubations are carried out in media prepared with 30 mM TRIS/30 mM histidine buffers. In these experiments the pH is adjusted to 8.5 before 5'-nucleotidase treatment. Incubation times are 30 min (Fig. 25a) or 10 min (Fig. 25b).

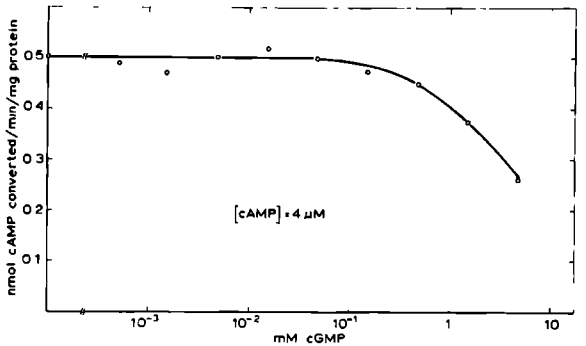

Fig. 26. Inhibition of 3',5'-AMP phosphodiesterase in rat pancreas by 3',5'-GMP. Diluted homogenate, containing 0.32 mg protein per ml, is incubated for 10 minutes in media containing 4 μM 3',5'-AMP and varying concentrations of 3',5'-GMP.

Effect of 3',5'-GMP

Addition to the incubation medium of 3',5'-GMP in concentrations up to 100 μM does not influence the rate of hydrolysis of low concentrations (1.3 - 20 μM) of 3',5'-AMP. Only at higher concentrations of 3',5'-GMP (1 mM), significant inhibition of 3',5'-AMP hydrolysis occurs (Fig. 26).

Effects of methylxanthines and papaverine

The methylxanthines theophylline and caffeine are known inhibitors of 3',5'-AMP phosphodiesterase activity. More recently, papaverine has been reported to be a more potent inhibitor than the methylxanthines (Pöch, 1971; Schwabe et al., 1972; Triner et al., 1970). The two methylxanthines and papaverine have, therefore, been tested for possible inhibition of 3',5'-AMP hydrolysis by rat pancreas homogen-

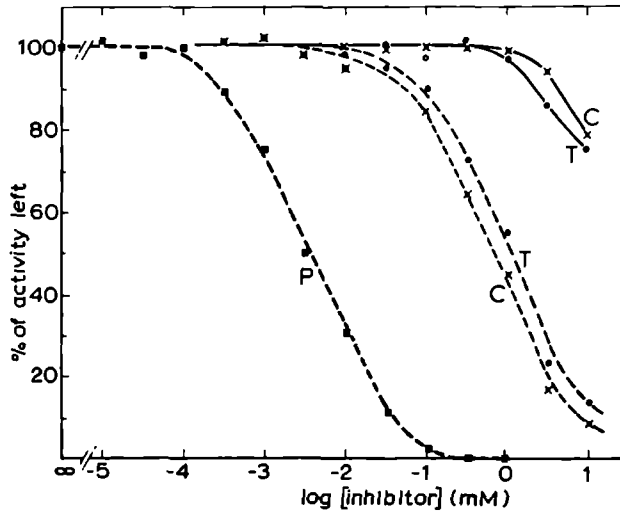

Fig. 27. Inhibition of 3',5'-AMP phosphodiesterase in rat pancreas by caffeine, theophylline and papaverine. Homogenate dilutions contain 25 mg protein per ml for 30 min incubations at 2 mM 3',5'-AMP concentration (solid lines) in the presence of varying amounts of caffeine (C), theophylline (T) or papaverine (P). At 4 µM 3',5'-AMP concentration (broken lines) the homogenate dilution contains 0.8 mg protein per ml for 10 min incubations.

ates. Both methylxanthines cause virtually equal inhibition of the two 3',5'-AMP phosphodiesterase activities (Fig. 27). The low K_m activity (half inhibition at 1 mM) is much more sensitive than the high K_m activity (half inhibition at > 10 mM). Papaverine is a much stronger inhibitor with half maximal inhibition at 3 µM for the low K_m activity, but it

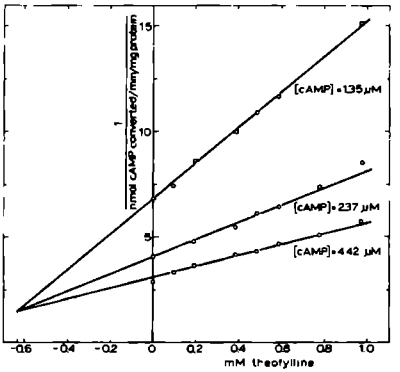

Fig. 28. Dixon-plot of the inhibition of 3',5'-AMP phosphodiesterase in rat pancreas by theophylline. The diluted homogenate contains 0.35 mg protein per ml. Incubations are carried out for 10 minutes.

again inhibits the low K_m activity at much higher concentrations (half inhibition at 2 mM). A Dixon plot of the theophylline inhibition of the low K_m activity (Fig. 28) demonstrates that the inhibition is of the competitive type (K_i = 0.63 mM). Papaverine (not shown) also inhibits the low K_m activity in a competitive way (K_i = 3 µM).

Effect of secretory stimulants

No change in either of the two 3',5'-AMP phosphodiesterase activities is observed, when carbachol (10 µM), secretin (10 units per ml) or pancreozymin-octapeptide (300 units per ml) are added to the incubation medium.

Solubility of the 3',5'-AMP phosphodiesterase activities

The filtered homogenate is centrifuged for 1 hour at 225,000 x g_{max} at 0°C in a Spinco Ti-50 rotor, and the resulting sediment is washed twice with 0.9% NaCl. The enzyme activity of the washed sediment is determined and compared with that of the homogenate. The 3',5'-AMP phosphodiesterase activities of the sediment at 4 µM and 2 mM substrate concentration amount to 21 and 16%, respectively, of the corresponding activity in the homogenate. This indicates that the major part of the two enzyme activities is particulate.

5.4. DISCUSSION

The 3',5'-AMP phosphodiesterase activity of rat pancreas has been characterized. In the present experiments total pancreas homogenate has been used, notwithstanding the fact, that this organ contains different cell types. Since the endocrine (islet) tissue comprises only 4% of the organ (Hegre et al., 1972), the results will represent primarely the enzyme occurring in the exocrine tissue, unless the islet cells would have a very high specific activity. Our finding that about 20% of both activities can be sedimented by 225,000 x g centrifugation also needs to be taken into account in further experiments aimed at identifying the location of the enzyme activities.

Our results suggest that two phosphodiesterase activities are present in rat pancreas, one with a high and the other with a low substrate affinity. This phenomenon has already been reported in a number of other rat tissues as brain (Thompson and Appleman, 1971a), adipose tissue (Loten and Sneyd, 1970; Thompson and Appleman,

TABLE VII

K_m VALUES FOR 3',5'-AMP OF PHOSPHODIESTERASES IN VARIOUS RAT TISSUES

K_m values for 3',5'-AMP of phosphodiesterases from various rat tissues as reported in literature are listed below and compared with the values obtained for rat pancreas.

Tissue	Low K_m, µM	High K_m, µM	Reference
Brain	5.0	104	Thompson and Appleman, 1971a
"	1.0	130	Brooker et al., 1968
Kidney	-	290	Dousa and Rychlik, 1970
"	2.7	95	Jard and Bernard, 1970
Heart	-	300	Therriault and Winters, 1970
Adipose tissue	0.9	41	Loten and Sneyd, 1970
" "	1.7	404	Klotz et al., 1972
Thymic lymphocytes	0.9	8	Franks and MacManus, 1971
Pancreas	4.9	367	This study

1971b; Klotz et al., 1972), kidney (Jard and Bernard, 1970) and thymic lymphocytes (Franks and MacManus, 1971), and also in tissues of other species as mouse pancreatic islets (Ashcroft et al., 1972), rabbit skeletal muscle (Huang and Kemp, 1971) and pig cerebrum (Weiss et al., 1972). The kinetic analysis of the data in these reports is not always adequate. In several cases the kinetic parameters K_m and V_{max} are simply obtained by plotting 1/v versus 1/[S]or v versus v/[S] (Huang and Kemp, 1971; Klotz et al., 1972; Ashcroft et al., 1972; Jard and Bernard, 1970; Franks and MacManus, 1971; Weiss et al., 1972). Such a simplification can lead to considerable errors, as has been shown by Spears et al. (1971). It will only give correct values, when both the K_m and V_{max} values differ greatly. When the V_{max} values are not greatly different, reasonably accurate values can be obtained without correction for the low K_m activity only (see equations (2) and (3) in section 5.2.).

Accurate values for the other activity can only be obtained by correcting the rates at high substrate concentrations for the contribution of the low K_m activity. The accuracy of the resulting values for K_m and V_{max} can be further improved by repeated cross-correction.

The K_m values for rat pancreas (4.9 and 267 μM) are of the same order of magnitude as found in other rat tissues, as is shown in Table VII. In rat adipose tissue three different phosphodiesterase fractions were separated, each of which showed different kinetic characteristics (Klotz et al., 1972; Thompson et al., 1971b).

In rat pancreas maximal phosphodiesterase activity is reached at lower magnesium concentrations (0.5 and 0.75 mM for low K_m and high K_m activities, respectively) than

reported in other tissues, where magnesium concentrations of over 1 mM are necessary, as in rat brain (Kakiuchi and Yamazaki, 1970b), rat kidney (Dousa and Rychlík, 1970) and dog heart (Nair, 1966). Only Huang and Kemp (1971) report full activation in rabbit skeletal muscle by 0.1 mM Mg^{2+}.

Calcium, in low concentrations, stimulates phosphodiesterase activity in rat brain in the presence of magnesium ions (Kakiuchi and Yamazaki, 1970a, 1970b; Kakiuchi et al., 1971; Miki and Yoshida, 1972), but such a stimulation has not been found in other rat tissues (Miki and Yoshida, 1972). We find that Ca^{2+} does not stimulate phosphodiesterase in rat pancreas, and has an inhibitory effect on the Mg^{2+} -stimulated activity, when added in concentrations above 0.1 mM.

The 3',5'-AMP phosphodiesterase of many tissues is able to hydrolyse 3',5'-GMP: rat brain (Kakiuchi et al., 1971; Thompson and Appleman, 1971a), rat adipose tissue (Klotz et al., 1972), rabbit skeletal muscle (Huang and Kemp, 1971), human thrombocytes (Song and Cheung, 1971) and several rat and bovine tissues (Beavo et al., 1970). In several of these cases this nucleotide also competitively inhibits the hydrolysis of 3',5'-AMP (Thompson and Appleman, 1971a; Klotz et al., 1972; Beavo et al., 1970; Franks and MacManus, 1971). In rat liver, however, 0.08 - 50 µM 3',5'-GMP stimulates 3',5'-AMP hydrolysis (initial 3',5'-AMP concentration: 1 µM), while it inhibits at concentrations above 50 µM (Beavo et al., 1971). In rat thymic lymphocytes the same phenomenon has been observed (Franks and MacManus, 1971). In rat pancreas homogenate the hydrolysis of 4 µM 3',5'-AMP is not influenced by 3',5'-GMP in concentrations below 100 µM, while higher concentrations

become inhibitory.

The inhibition of 3',5'-AMP phosphodiesterase by methylxanthines and by papaverine is a well-known phenomenon. The finding that the inhibition by theophylline is of the competitive type is also in agreement with an earlier report concerning rat adipose tissue (Schwabe et al., 1972). For papaverine competitive inhibition has been described in rat adipose tissue (Schwabe et al., 1972) and rat myocardial and coronary artery supernatant (Pöch et al., 1971), while non-competitive inhibition was found with purified beef heart phosphodiesterase (Pöch et al., 1971). The relative potencies of papaverine and theophylline for pancreas are approximately the same as in rat adipose tissue (Schwabe et al., 1972), rat myocardial tissue and coronary artery (Pöch, 1971) and in rabbit aorta (Triner et al., 1970).

In conclusion, some remarks about the physiological significance of these findings are in order. The low K_m activity would seem to be more important than the high K_m activity for the physiological events in the pancreas, since the 3',5'-AMP levels within the resting and the stimulated cat and guinea-pig pancreas vary between 0.3 and 10 µmoles/kg (Case et al., 1972; Benz et al., 1972). The high K_m activity may function as a safeguard against incidentally and locally higher concentrations of 3',5'-AMP inside the cell, or it may represent a phosphodiesterase specific towards other nucleotides. Another possibility would be that the two activities are located in different types of cells of the pancreas. The total capacity of the phosphodiesterase system is very large compared to that of the adenylate cyclase system, even when the latter is stimulated maximally. From the data in Chapter III it can be calculated

that the rate of hydrolysis at a 3',5'-AMP concentration of 4 µM is about 50 times that of its rate of formation. This finding may explain why the 3',5'-AMP levels in isolated cat pancreas rise only very briefly after stimulation (Case et al., 1972).

When it is true that stimulation of the pancreatic enzyme secretion is mediated by 3',5'-AMP, the relatively high potency of papverine as an inhibitor of phosphodiesterase agrees with the observation that in the isolated rabbit pancreas papaverine is a more potent stimulant of enzyme secretion than theophylline, as described in section 2.3. The finding that at a 3',5'-AMP concentration of 4 µM the activity of the enzyme in the presence of 10 mM theophylline is still 10% of the activity in the absence of this compound could also offer an explanation for the observation that theophylline in concentrations of up to 10 mM has hardly any effect on the enzyme secretion by the isolated organ. The rate of decomposition of the nucleotide in that case still exceeds the maximal rate of formation several times. Papaverine in a concentration of 1 mM, however, causes complete inhibition of the enzyme and therefore can be expected to stimulate enzyme secretion, as we have indeed observed (section 2.3.).

CHAPTER VI

EFFECTS OF CYCLIC AMP ON PANCREATIC LIPOLYTIC ENZYMES. STABILITY STUDIES ON ISOLATED ZYMOGEN GRANULES FROM PANCREAS AND ON ERYTHROCYTES

6.1. INTRODUCTION

In Chapter I the morphological and physiological features of the enzyme secretion process have been described. This process is not limited to the acinar cells of the pancreas, but a similar mechanism can be observed in most, if not all of the cell types, which secrete large amounts of high molecular weight peptides and proteins. Some of these processes are listed in Table VIII.

In the previous chapter it has already been stated that most of these processes have also in common the fact that cyclic AMP plays an intermediate role in the stimulus-secretion process. However, in none of these cases has the way in which cyclic AMP performs its function, yet been elucidated. It could very well be that this mechanism would be the same for all these cells. Thus, the hypothesis of Ridderstap and Bonting (1969c), described in section 1.4., if applicable for the enzyme secretion by the pancreas, might also apply to these other secretion processes. Hence, it is of considerable importance to test the validity of this hypothesis. For this reason we have studied the effect of cyclic AMP on the phospholipase and lipase activities of the pancreas. The results are described in this chapter.

There may, however, be other ways, by which cyclic AMP

TABLE VIII

BULK SECRETORY PROCESSES

Organ	Stimulus	Secreted substance(s)
Parotid gland	catecholamines	amylase
Pancreas	catecholamines glucagon corticotrophin glucose	insulin
Pancreas	amino acids	glucagon
Thyroid gland	thyrotrophin	thyroid hormones
Thyroid gland	Ca^{2+} glucagon	calcitonin
Anterior pituitary	releasing hormones from hypothalamus	corticotrophin thyrothrophin growth hormone prolactin gonadotrophic hormones

plays its intermediate role. A more immediate effect of the nucleotide in the fusion of cellular and granular membranes cannot be excluded. A high intracellular level of the compound might introduce physical changes in the membranes, which could induce fusion. To test this possibility the effects of cyclic AMP on the stability of zymogen granules in suspension and also on a model system, viz. erythrocytes, have been studied. Since we have described in Chapter VII, that lysophospholipids are an important component of the granular membrane or else may very easily be formed, the possible interaction of lysophosphatidylcholine and cyclic AMP on erythrocyte lysis has also been investigated.

6.2. METHODS

Isolation of zymogen granules from pig pancreas

Pig pancreas is obtained approximately 15 min after death of the animal and is transported on ice to the laboratory. Within 30 minutes after death fat and connective tissue are removed and about 100 - 150 g of glandular tissue is homogenized for 20 seconds in a Waring blendor in 3 vols ice-cold 0.3 M sucrose. The homogenate is filtered through four layers of medical gauze and then centrifuged for 10 minutes at 3300 g_{max} in a Sorvall RC2B centrifuge. The upper fluffy layer of the pellet is removed by gentle swirling with 0.3 M cold sucrose.

. The remaining pellet is resuspended in 30 ml of cold 0.3 M sucrose and centrifuged for 5 minutes at 370 g_{max}. The resulting pellet, consisting mainly of erythrocytes, nuclei and debris, is discarded and the supernatant is recentrifuged at 1475 g_{max} for 10 minutes at $0^{o}C$. The

resulting white pellet consists mainly of zymogen granules. The fluffy brown layer of mitochondria on top of the granule pellet can be removed by gentle rinsing and swirling with cold 0.3 M sucrose. The fraction is then further purified by repeated centrifugation at 370 and 1475 g_{max}.

Stability studies on isolated zymogen granules

Zymogen granules, isolated and purified as described above, are resuspended in 0.3 M sucrose, buffered with ammonium acetate - imidazole (5 mM each), brought to pH 5.6 with HCl. This suspension is diluted with the same solution, so that the suspension has an extinction at 625 nm of approximately 1.0 (1 cm cuvet). Lysis of the zymogen granules at 20°C is measured by monitoring the decrease in absorbance at this wavelength. Additions are made up in concentrated form with the same solution, while the pH of the resulting solutions is brought to 5.6.

Lipase and phospholipase activities

Lipase activity is determined by the method of Marchis-Mouren et al. (1959), using an emulsion of olive oil as substrate. A unit of activity is defined as 1 µmol free fatty acid released per minute at 37°C.

Phospholipase A is assayed according to De Haas et al. (1968a). An egg yolk emulsion is used as substrate and the enzyme activity is measured by automatic titration of the liberated fatty acids at pH 8.0 on a Radiometer pH-stat TTT 1. The Ca^{2+} concentration is maintained at 6 mM, while sodium deoxycholate is present in a 2.7 mM concentration. A unit of activity is defined as 1 µmol free fatty acid released per minute at 37°C.

Experiments with pig erythrocytes

Pig erythrocytes, prepared from fresh blood, are washed 3 times with 0.9% NaCl and then diluted with 0.9% NaCl to a suspension, which upon 60-fold dilution with the same solution has an optical density of approximately 1.5 at 625 nm (1 cm cuvet). Stability studies are done by adding to 3.00 ml of buffered 0.9% NaCl in a 1 cm cuvet 10 µl of an aqueous solution of lysophosphatidylcholine or other compound and immediately thereafter 50 µl of the erythrocyte suspension. The decrease of the optical density in dependence of time is then recorded.

6.3. RESULTS

Isolation of zymogen granules

A pure zymogen granule fraction is best obtained by differential centrifugation (Meldolesi et al., 1971a). The combined differential and gradient centrifugation procedure for ox pancreas of White and Hawthorne (1970), who first prepared a mixed pellet of zymogen granules and mitochondria which is then separated by gradient centrifugation of the pellet, does not give a satisfactory separation of zymogen granules from mitochondria of pig pancreas in our hands. This is determined by assay of the activities of amylase and succinate dehydrogenase as marker enzymes for zymogen granules and mitochondria, respectively (see section 7.3.). Therefore, we have used the procedure as described in section 6.2. Electron micrographs of the zymogen granule fraction (Fig. 29) show the typical picture of electron-dense round bodies with only slight contamination by other structures, as also found by Meldolesi et al.,(1971a).

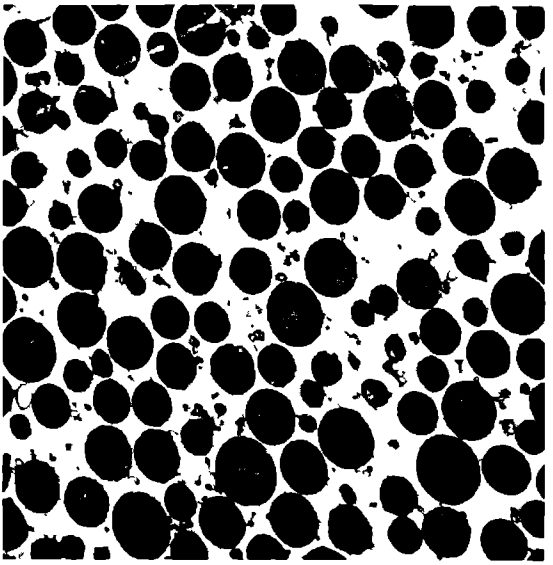

Fig. 29. Electronmicrograph of zymogen granule fraction. Pellets are fixed in 2% glutaraldehyde, followed by 1% OsO_4, both in 0.1 M cacodylate buffer, pH 7.4. Parts of the pellets were dehydrated, embedded in Epon and thin sections are cut, which are stained with uranylacetate and lead citrate, and are examined in a Philips EM 200 electron microscope. Magnification: 14.500 x.

Effects of cyclic AMP on lipolytic enzymes

The phospholipase assay, as described by De Haas et al. (1968a), in our hands shows no linear characteristics. While these authors observe a proportionally increasing fatty acid production with regard to time, in our case activity increases with time of incubation. In addition we find in freshly prepared whole pancreas homogenates much higher activities than these authors.

TABLE IX

EFFECT OF CYCLIC AMP ON LIPOLYTIC ENZYMES IN PANCREAS HOMOGENATES

Aliquots of pig pancreatic homogenates in 0.3 sucrose are either preincubated at pH 7.0 in the presence of the stated additions followed by lipase and phospholipase assays, or directly assayed in the presence of the additions. The titrimetric assay methods applied in these experiments are described in section 6.2. Activities are given as mean values with the S.E. of the mean, or as the results of the separate experiments.

Additions	Lipase		Phospholipase A_2	
	direct	preincubated (10 min)	direct	preincubated (15-30 min)
No addition	100%	100%, 99%	100%	150%±15(4)
Cyclic AMP (1 mM)	93%, 95%	93%, 98%	95%±9(5)	151%±18(4)
ATP (1 mM), Mg^{2+} (1 mM)	100%,101%	102%,102%	-	153%
ATP (1 mM), Mg^{2+} (1 mM), cyclic AMP (1 mM)	93%, 95%	97%, 99%	-	126%
ATP (1 mM), Mg^{2+} (1 mM), supernatant (1 mg protein/ml)			-	148%
ATP (1 mM), Mg^{2+} (1 mM), cyclic AMP (1 mM), supernatant (1 mg/protein/ml)			-	143%

Cyclic AMP, present during the assay in concentrations up to 1 mM does not stimulate phospholipase A activity (Table IX). The same is true when preparations of zymogen granules are used instead of homogenate. Preincubation of homogenate or zymogen granules at pH 7.0 and 37° with cyclic AMP in concentrations up to 1 mM does not increase phospholipase A activities compared with preparations, preincubated without the nucleotide. However, always a remarkable, probably autolytic, activation of phospholipase A is noted. Addition of magnesium (1 mM) and ATP (1 mM) during the incubation or preincubation does not change these findings.

For lipase the results are essentially the same, except that no such autolytic activation is seen (Table IX). Addition of supernatant protein in combination with magnesium and ATP is again without effect on these results.

Effects of cyclic AMP on the stability of isolated zymogen granules

Suspensions of isolated zymogen granules show more or less the same characteristics as zymogen granules from dog pancreas, as described by Hokin (1955). Suspensions are stable at pH 5.5 - 5.6, at least when the turbidity of the suspensions is used as criterion. This can be seen in Fig. 30. At pH-values above 7 such suspensions almost completely clear up. Similar findings have been reported by Rothman (1971), who measures release of digestive enzymes at different pH values. In accordance with his findings, preparations in 0.3 M sucrose are more stable than those made in 0.9% NaCl or other electrolyte solutions. It must be noted that the various preparations differed also for no

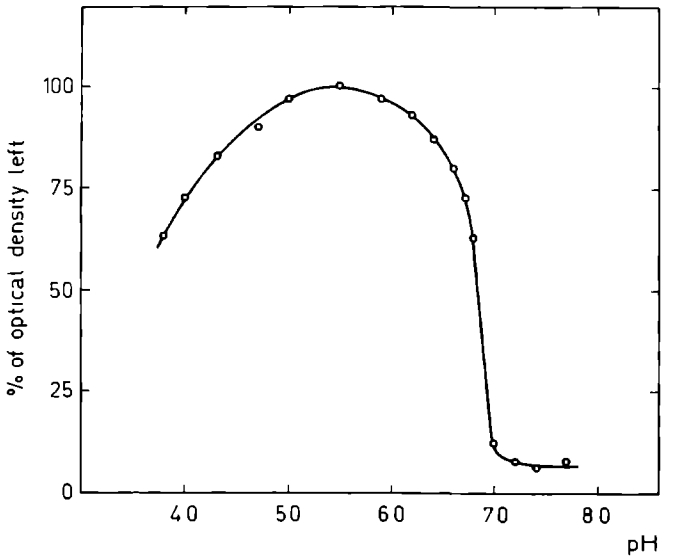

Fig. 30. Effect of pH on the stability of isolated zymogen granules. Zymogen granules are isolated as described in section 6.2. and resuspended in 0.3 M sucrose, buffered with ammonium acetate-imidazole (5 mM each), brought to the desired pH with HCl or NaOH. Values are expressed as percentage of initial optical density at 625 nm, left after 3 min.

apparent reason, in the rate of spontaneous lysis at pH 5.6. Storage of the suspensions at 0^{o} always leads to an accelerated decrease in turbidity.

Fig. 31 shows the effect of 1 mM cyclic AMP on the lysis of a freshly prepared zymogen granule suspension, as compared to a control preparation. It can be seen that the compound has a definite effect on the stability of the

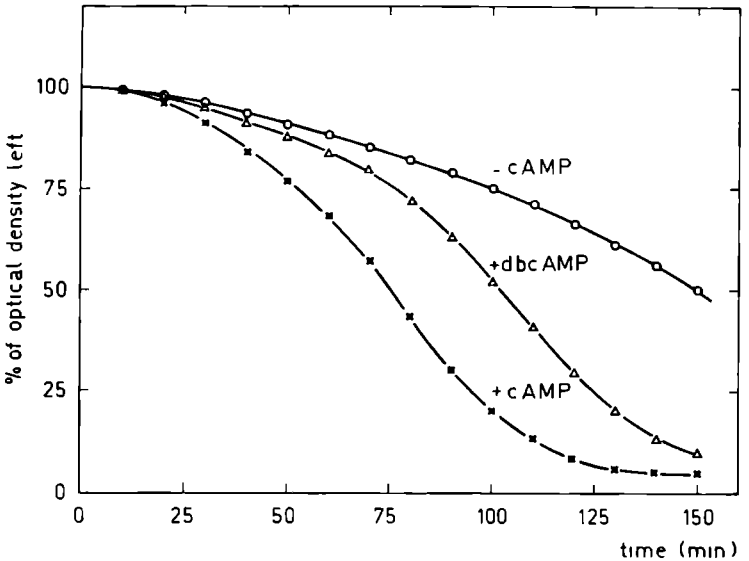

Fig. 31. Effect of cyclic AMP and its dibutyryl derivative on the stability of isolated zymogen granules. This is determined as described in section 6.2. Results are expressed as percentage of the initial optical density of the suspension at 625 nm.

o——o : no addition
x——x : 1 mM cyclic AMP present
Δ——Δ : 1 mM dibutyryl cyclic AMP present

granules, which becomes significant only after several minutes. The control preparation shows some spontaneous lysis. 5'-AMP does not have a similar effect. Simultaneous addition of 1 mM Ca^{2+} together with 1 mM cyclic AMP abolishes the effect of the nucleotide. Due to the different stability of the various preparations, the effect of the 1 mM cyclic AMP is not always quantitatively the same, but qualitatively it is reproducible. In lower concentration the nucleotide

(0.1 mM) causes some lysis only in a few cases. The dibutyryl derivative of cyclic AMP also causes lysis in a concentration of 1 mM, but its effect is smaller than that of cyclic AMP.

Experiments with pig erythrocytes

It is known that ox erythrocytes are lysed by various lysolecithins and lecithins (Reman et al., 1969). The same

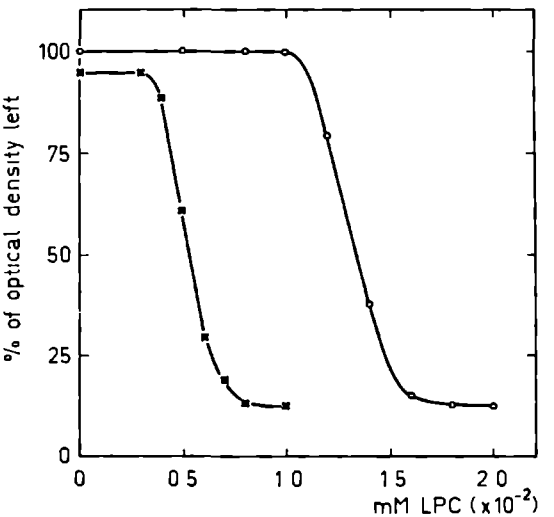

Fig. 32. Effect of lysophosphatidylcholine alone and in combination with secretin on the stability of erythrocytes.
Erythrocytes are prepared as described in section 6.2. and resuspended in 0.9% saline, buffered with 10 mM TRIS-HCl, pH 7.5. The experiments are carried out as described under Methods. Results are expressed as percentage of initial optical density at 625 nm left after 3 min vs. lysophosphatidylcholine concentration.

o——o : no secretin present
x——x : $3x10^{-2}$ mg/ml secretin ($3x10^{-2}$ clinical unit/ml) present.

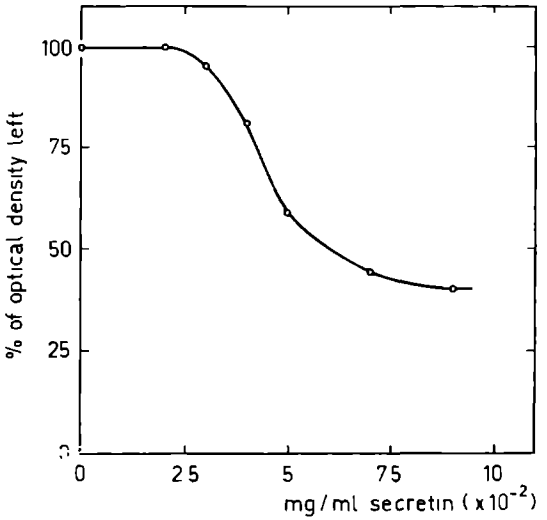

Fig. 33. Effects of a secretin preparation (BOOTS, specific activity 1 clinical unit/mg) on the stability of erythrocytes. For further explanation, see legend to Fig. 32.

is true for pig erythrocytes, as shown in Fig. 32. The above mentioned authors measured the time required to cause a 50% drop in transmission at 625 nm. Since we have observed that after addition of lysophosphatidylcholine the optical density of the suspension decreases sharply in the first minute, but thereafter remains relatively constant, we have plotted percentage of initial optical density left after 3 minutes vs. lysophosphatidylcholine concentrations.

Next, we have tested, whether cyclic AMP is able to potentiate the lytic action of lysophosphatidylcholine on the erythrocytes. This is done at pH values of 6.0 to 8.0, in or without the presence of 10^{-5} to 10^{-3} M Ca^{2+}. In none of these circumstances, however, does cyclic AMP in concentra-

tions up to 1 mM cause any shift in the curve (Fig. 32).

When instead of cyclic AMP low concentrations of commercial secretin preparations are added, the lysis curve shifts to lower lysophosphatidylcholine concentrations (Fig. 32). Commercial preparations of pancreozymin show the same phenomenon. Different batches of the commercial hormones varied quantitatively in their effect.

Further analysis of this effect indicates that also without addition of lysophosphatidylcholine these hormones, though in somewhat higher concentrations, cause substantial lysis of erythrocytes. For one batch of secretin this is shown in Fig. 33. Although sublytical concentrations of the hormones, combined with sublytical concentrations of lysophosphatidylcholine, cause lysis of pig erthrocytes, it can probably be concluded that this is merely an additive effect instead of a potentiating one, because it was also found that synthetic secretin lacks this effect.

Other hormones, such as glucagon, insulin, synthetic vasopressin and oxytocin have also been tested for such effects, but with negative results.

6.4. DISCUSSION

In the first part of this chapter it is reported that at least under the conditions used, no in vitro stimulation of pancreatic phospholipase A or lipase by cyclic AMP can be demonstrated. Studies on pancreatic lipase by other authors seem to point in an opposite direction. Santhanam and Wagle (1971) have reported a stimulation of pancreatic lipase by cyclic AMP of up to 60% after preincubation in the presence of Mg^{2+} and ATP. For fat cell phospholipase A a 3-fold stimulation in the presence of 2 mM cyclic AMP is

reported (Chiappe de Cingolani et al., 1972). In rat adipose tissue homogenates cyclic AMP in micromolar concentrations stimulates fatty acid liberation from endogenous triglycerides in the presence of Mg^{2+} up to 2-fold (Wade, 1970).

Our negative results do not support the hypothesis of Ridderstap that cyclic AMP might influence pancreatic enzyme secretion by stimulating the formation of endogenous lysophosphatides. The lipolytic enzymes in the pancreas probably have only digestive functions, in which respect they differ from the enzymes in adipose tissue. However, it must be admitted that small amounts of (phospho) lipases with a more specific metabolic function might go unnoticed in the assay because of the overwhelming amounts of the digestive lipolytic enzymes. Our findings, therefore, may not be taken as a definitive disqualification of the hypothesis of Ridderstap and Bonting (1969c).

Another possibility would be that cyclic AMP directly influences emiocytosis without intermediate steps of enzyme activation, but merely through a physico-chemical effect on membrane structure. Such a hypothesis could easily be fitted in the model of membrane fusion as proposed by Lucy and others (Howell and Lucy, 1969; Lucy, 1970; Ahkong et al., 1972). For this reason the effect of cyclic AMP on the stability properties of isolated zymogen granules has been studied.

Already in 1955 it has been observed that dog pancreatic zymogen granules release their protein in solutions with a pH above 7. This is accompanied by a decrease in the turbidity of the suspension (Hokin, 1955). We find the same to be true for zymogen granules from pig pancreas. More recently, Rothman (1971) has reported similar observations

for zymogen granules from rat pancreas, except that the various enzymes present in the zymogen granules are not all released at the same rate. It is of interest that for zymogen granules from parotid gland such a pH-dependent stability has never been reported.

One observation we have made is that zymogen granules suspended in isotonic saline at pH 5.6 are rather unstable. In a more recent publication this is also found for rat pancreatic zymogen granules (Burwen and Rothman, 1972). The same is true for all electrolyte solutions they studied. In fact, protein release is dependent on the osmolarity of the solution. If the suspension is prepared with isosmotic solutions of non-electrolytes, the zymogen granules are stable. For zymogen granules from parotid gland this does not seem to be the case (Ishida et al., 1971a, 1971b). It is of interest that adreno-medullary storage granules are most stable at half-isotonic electrolyte concentrations in a sucrose medium (Lishajko, 1971).

As shown in the Results section, cyclic AMP increases the lysis rate of pancreatic zymogen granules, although only at relatively high concentration (1 mM) and after a relatively long incubation time. The effect is rather specific, not being given by AMP and only a little by the dibutyryl derivative of cyclic AMP. It seems unlikely that the compound would also act in vivo as a membrane destabilizing agent, because in vivo such high cyclic AMP concentrations are never found.

It is interesting that calcium (1mM) prevents the lysis by cyclic AMP. This seems to be somewhat in contrast with the findings of Burwen and Rothman (1972) that zymogen granules from rat pancreas, suspended in isotonic $CaCl_2$, release

most of their enzymes. However, that observation does not refer to low concentrations of Ca^{2+} in isotonic sucrose. Rat parotid zymogen granules, suspended in an isotonic medium with K^{+} as the major cation, release amylase upon addition of Ca^{2+} in concentrations of 0.2 mM or higher, but only if Mg^{2+} and ATP are present in millimolar concentrations (Ishida et al., 1971b). Adreno-medullary storage granules, suspended in isotonic saline which also contains 10 mM potassium phosphate release catecholamines upon addition of 2 mM Ca^{2+}, but when they are suspended in isotonic sucrose, Ca^{2+} inhibits release. The matter becomes even more complex by the fact that stimulation by Ca^{2+} in saline medium is blocked by the addition of Mg^{2+} and ATP (Lishajko, 1971).

In the membranes of adreno-medullary storage granules large amounts of lysophosphatidylcholine are found (Blaschko et al., 1967). As we will describe in chapter VII, we have made the same observation for zymogen granules from pig pancreas. This has led us to study how cyclic AMP and some other compounds affect the stability of a model membrane system. We have chosen erythrocytes for the reason that they are easy to handle and that like artificial phospholipid membrane systems, they are highly susceptible to the lytic action of lysophospholipids. We have first examined the effect of lysophosphatidylcholine on the turbidity of erythrocyte suspensions. The earlier observations of Reman et al (1969) have been confirmed, except for the fact that these authors found a certain "lag time" before start of hemolysis. Therefore they related lytic agent concentration to the time needed for 50% hemolysis. We find that hemolysis after 3 minutes affords a better parameter for lytic action, because the hemolysis rate is fast in the first min-

ute and thereafter slows down until after about 2 minutes there is no further increase in hemolysis.

Cyclic AMP has no effect on the relation between degree of hemolysis and lysophosphatidylcholine concentration, indicating that in this system the nucleotide does not show a membrane labilizing effect. Addition of commercial preparations of pancreozymin and secretin, however, causes a shift in the observed curve to lower phosphatidylcholine concentrations (Fig. 32). This raises the question, whether they could act similarly in vivo on zymogen granules. This seems unlikely, because these polypeptide hormones, consisting of about 30 amino acids, are not expected to enter the acinar cells. These hormone preparations by themselves also cause erythrocyte lysis, so that in fact they have no potentiating but only an additive effect. These preparations are of rather low degree of purity, having specific activities only of a few units per mg while pure synthetic preparations have specific activities of several thousand units per mg. Hence, it seems likely that the effect is not caused by the hormone, but by accompanying impurities in the preparations.

The conclusion from the experiments in this chapter must be that no evidence for the validity of the hypothesis of Ridderstap and Bonting (1969c) has been found.

The peculiar stability characteristics of pancreatic zymogen granules are most probably not only caused by the phospholipid composition of their membranes, but also by the manner in which the proteins and enzymes are stored in the granules. They cannot be present as free molecules, since their high concentration would then cause osmotic lysis of the granule. Therefore these proteins may be associated in

osmotically inactive complexes, which may be broken up by e.g. changes in pH. This point is also discussed by Burwen and Rothman (1972), who in support of this assumption find reaggregation of the solubilized enzymes under certain conditions. This would also offer an explanation for the fact that parotid zymogen granules do not behave in a similar manner as granules from pancreas. The major difference between both types of granules is in their protein content, granules from pancreas containing a mixture of different enzymes, while in parotid granules α-amylase is almost the only constituent of the content.

CHAPTER VII

PHOSPHOLIPIDS OF THE PANCREAS

7.1. INTRODUCTION

When the pancreatic acinar cell extrudes its zymogens by emiocytosis, the membranes of the zymogen granules fuse with the apical plasma membrane, forming a hole through which the granule contents enter the lumen. It seems reasonable to assume that knowledge of the composition of these membranes, particularly the phospholipid composition, is essential for our understanding of the mechanism of this fusion process. An attractive model for such a fusion process has been advanced by Lucy (1970), who proposes that certain chemical agents like lysophospholipids may be incorporated into a "dynamic" membrane, so that little is needed to cause structural changes, by which membrane fusion is made possible. LPC is indeed able to cause cell fusion without loss of cell viability (Ahkong et al., 1972). Moreover, in the adrenal medulla, which shows bulk release by emiocytosis, the membranes of the amine containing storage granules contain large amounts of LPC (Blaschko et al., 1967).

There is another observation, which pleads for a role of phospholipids in emiocytosis: the so-called "phospholipid effect", first reported by Hokin and Hokin (1953). They

For reasons of convenience in this chapter the following abbreviations will be used: LPC = lysophosphatidylcholine; PC = phosphatidylcholine; LPE = lysophosphatidylethanolamine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PA = phosphatidic acid; PS = phosphatidylserine.

observed that secretion stimulation by acetylcholine and pancreozymin greatly increases ^{32}P incorporation in pancreatic phospholipids, especially PI and PE. Under certain circumstances this effect can be dissociated from secretion (Hokin, 1966; Bauduin and Cantraine, 1972). Similar "phospholipid effects" have also been reported for other cell-types, e.g. adrenal medulla (Hokin et al., 1958) and parotid gland (Hokin and Sherwin, 1957).

These considerations have led us to a study of the phospholipids of the pig pancreas. The results of this study are reported in this chapter.

7.2. METHODS

Preparation of subcellular fractions from pig pancreas

Zymogen granules are obtained as described in section 6.2. For the isolation of the other subcellular fractions the first 3300 g_{max} supernatant is recombined with the upper fluffy layer of the pellet.

The mitochondrial fraction is isolated by centrifuging this supernatant for 10 min at $0^{o}C$ at 12,000 g_{max} in a Sorvall RC2B centrifuge. The resulting pellet, which consists of mitochondria contaminated with zymogen granules and microsomes, is purified by repeated contrifugation at 1475 and 12,000 g_{max}. Our best mitochondrial preparations are still contaminated by zymogen granules, which is shown by the fact that the specific amylase activity in this fraction is 14% of that in the zymogen granule fraction (Table X).

The microsomal fraction is prepared by centrifuging the mitochondrial supernatant for 120 min at 105,000 g_{max} at $0^{o}C$

in a Spinco 30 rotor and is not further purified. The resulting supernatant is further referred to as PMS (post microsomal supernatant).

Membranes of the zymogen granules are isolated in the following way. The purified zymogen granule pellet is suspended in 15 ml of icecold 0.9% NaCl, containing 10 mM TRIS at pH 8.0 and 0.5 mg/ml soybean trypsin inhibitor in order to lyse the granules. The clear suspension is centrifuged for 120 min at 0^{o}C in a Spinco T150 rotor at 225,000 g_{max}. The resulting pellet is then resuspended in 3 - 4 ml of the same solution.

For phospholipid analysis all fractions are used without further treatment. For most other purposes they are resuspended in 0.3 M sucrose and stored at -70^{o}C when not used immediately.

Phospholipid extraction and analysis

Complete lipid extraction from small amounts of fresh filtered homogenate or subcellular fractions is performed essentially according to the method of Folch et al. (1957). The resulting washed lipid extract in chloroform-methanol (2 : 1, by vol.) is concentrated by evaporation and dissolved in a known volume of benzene-ethanol (4 : 1, by vol.) and stored at -20^{o}C under nitrogen.

Phospholipids in these extracts are separated according to Broekhuyse (1968) by two-dimensional chromatography on 0.3 mm thick layers of purified silicagel H (Merck) or silicagel HR (Merck) containing 4% alkaline magnesium silicate (Woelm). The thin layers are developed in the first dimension with chloroform-methanol-7 N ammonia (90 : 54 : 11, by vol.) and after drying in vacuo over concentrated

H_2SO_4 in the second direction with chloroform-methanol-glacial acetic acid-water (90 : 40 : 12 : 2, by vol.). The lipid spots are visualized by means of exposure to iodine vapour and after evaporation of iodine free amino groups are detected with ninhydrin-reagent (Skidmore and Entenman, 1962).

Each spot is then scraped off quantitatively and the phosphorus content determined after digestion in 0.2 ml concentrated H_2SO_4 -70% $HClO_4$ (9 : 1, by vol.) by means of a modified Fiske-Subbarow method (Broekhuyse, 1968).

Experiments with radioactive phosphatidylcholine

Radioactive PC (^3H-labeled in the 1-acyl or ^{14}C-labeled in the 2-acyl fatty acid) is incubated with different subcellular fractions in order to obtain information about possible breakdown of phospholipids under isolation conditions. A known amount of radioactive PC (a mixture of 150 nmol, 5,000 cpm ^3H-labeled and 3.3 nmol, 10,000 cpm ^{14}C labeled) is suspended by vigorous mixing in 0.25 ml icecold 0.3 M sucrose. To this suspension 0.25 ml subcellular fraction is added and after incubation total lipids are extracted from this mixture as described before. The extract is concentrated and dissolved in a known volume of benzene-ethanol (4 : 1, by vol.). PC, LPC and free fatty acids are separated by thin layer chromatography according to Skipski et al. (1964). Samples of known volume of the lipid extract are spotted on 0.3 mm layers of silicagel (Merck) together with a solution of unlabeled PC, LPC and oleic acid. The thin layers are developed with chloroform-methanol-acetic acid-water (25 : 15 : 4 : 2, by vol.). After visualizing the spots by means of exposure to iodine vapour, they are scraped off on filter paper (Ø 4 cm). The filter papers and their

contents are burned in a Packard Tri-Carb Sample Oxidizer, the resulting $^{3}H_2O$ and $^{14}CO_2$ being captured separately and automatically in counting vials with scintillation solution. The separation of the isotopes is excellent, and also the recovery of ^{3}H and ^{14}C is virtually complete. Radioactivity is counted in a liquid scintillation spectrometer (Packard Tri-Carb, model 3380).

Analysis of fatty acid residues in phospholipids

A sample of lipid extract is subjected to the two-dimensional thin layer chromatography procedure described above. The spots are scraped off after visualization with Rhodamine-6G and the phospholipids are eluted three times with a mixture of chloroform-methanol (2 : 1, by vol.). After evaporation of the solvent, the residue is redissolved in benzene-ethanol (4 : 1, by vol.) and stored at -20°C under nitrogen.

Preparations of fatty acid methylesters for gas liquid chromatography is carried out with boron trifluoride (Morrison and Smith, 1964). The resulting fatty acid methylesters are stored in pentane at -20°C. They are separated over a column of 10 or 15% diethylene-glycol succinate on Gas Chrom P (Applied Science) at 180°C.

Other assay methods

Protein is measured as described in section 2.3.

Amylase activity is determined by the method of Bernfeld (1955), using soluble starch (p.a. Merck) as substrate, except that the optical density of the resulting solution is measured at 540 nm instead of 510 nm after 1 min and 4 min of incubation time. The difference between the

colorimeter readings obtained at 1 min and 4 min gives a better value for the activity than the difference between readings at 0 and 3 min. One unit of activity is defined as 1 mg maltose liberated in 3 min at 30°C.

Succinate dehydrogenase is determined according to King (1967), using phenazine methosulfate as an artifical electron acceptor. A unit of activity is defined as a 0.001 decrease in extinction at 600 nm in 2 min at 20°C.

Lipase assays are performed as described in section 6.2.

Lysophospholipase is assayed as described by Van den Bosch et al. (1968), using 1-[1-^{14}C]-palmitoylglycero-3-phosphorylcholine as substrate.

Phospholipase A_1 and A_2 are assayed using 1-[9,10-$^{3}H_2$]-palmitoyl-2-[1-^{14}C]-linoleoyl glycero-3-phosphorylethanolamine as substrate. The assay mixture contains 200 nmoles of the doubly-labeled phosphatidylethanolamine (1600 cpm ^{3}H and 1200 cpm ^{14}C), 100 µmoles of TRIS-maleate pH 7.0 and 1.0 µmole of $CaCl_2$ in a total volume of 0.5 ml. The reactions are stopped by starting a Dole-extraction procedure for fatty acids (Dole, 1956). Aliquots of the heptane extracts are chromatographed over small silicagel columns to free the fatty acids from contaminating phosphatidylethanolamine. The heptane eluates are collected in scintillation vials containing 16 ml of a toluene scintillation mixture. Radioactivity is determined in a Packard Tricarb liquid scintillation spectrometer.

It should be noted that this assay method gives maximum values for the overall release of fatty acids from the 1- and 2- position of phosphatidylethanolamine, rather than exact values for the phospholipase A_1 and A_2 activities.

TABLE X

DISTRIBUTION OF TWO MARKER ENZYMES OVER SUBCELLULAR FRACTIONS.

Fractions are isolated and enzyme activities are determined as described in section 7.2. Results are given as specific activities.

Tissue fraction	amylase (units/mg protein)	succinate dehydrogenase (units/mg protein)
Homogenate	90	112
Zymogen granules	189	47
Mitochondria	25	809
Microsomes	34	50
PMS	113	50

Isolation and characterization of subcellular fractions

The reasons for the choice of the differential centrifugation procedure have already been discussed in the previous chapter. The main reason is that gradient centrifugation does not yield reasonably pure zymogen granules and mitochondria.

The satisfactory purity of the fractions, obtained by this procedure, is shown by Table X, which gives the specific activities of amylase and succinate dehydrogenase serving as marker enzymes for zymogen granules and mitochondria, respectively. Another indication is the preferential localization of cardiolipin in the mitochondrial fraction, to be discussed in the next paragraph. Electron micrographs of the zymogen granule fraction show good purity, as discussed in section 6.3.

Lipid composition of pancreatic subcellular fractions

The phospholipid composition of the homogenate and the various particulate fractions and of zymogen granule membranes is shown in Table XI. PC and PE are the dominant phospholipids in all fractions. The microsomal fraction has the highest PC concentration. Cardiolipin is preferentially localized in the mitochondrial fraction. Its low concentration in other fractions parallels the distribution of succinate dehydrogenase activity. In contrast, sphingomyelin is most concentrated in the zymogen granules. As might be expected, the membranes of the zymogen granules have virtually the same phospholipid composition as the intact granules.

TABLE XI

PHOSPHOLIPID COMPOSITION OF FRACTIONS PREPARED FROM PIG PANCREAS

Aliquots of homogenate or subcellular fractions are extracted according to Folch et al.(1957). Phospholipid composition of the extracts is determined by thin layer chromatography. Technical details are given in section 7.2. Values, expressed as % of total lipid-P recovered from the thin-layer chromatograms, represent averages with S.E. of the mean. The number of experiments is given in parentheses at the top of the column.

Phospholipid	Zymogen granules (12)	Mitochondria (3)	Microsomes (4)	Zymogen granule membranes (2)	Whole homogenate (4)
Origin	0.2±0.1	-	0.3±0.1	1.0	0.2±0.2
Lyso-PC	12.8±1.1	8.3±2.3	1.4±0.5	12.1	0.3±0.3
PC	29.8±1.3	33.5±2.0	53.8±2.0	26.8	49.6±1.7
Lyso-PE	14.4±1.0	9.5±0.7	1.0±0.3	14.9	0.4±0.3
LPE	18.1±1.1	23.1±0.9	27.5±0.5	14.6	27.0±0.8
Sphingomyelin	10.0±0.5	2.2±0.4	1.7±0.1	9.2	4.1±0.6
PI + PA	9.2±0.5	10.0±0.4	9.9±0.3	14.7	8.8±0.3
PS	0.1±0.1	1.0±0.2	3.1±0.4	2.2	4.0±0.7
Cardiolipin	2.9±0.3	9.1±0.9	0.4±0.1	2.2	2.5±0.9
Front	1.9±0.5	1.8±0.7	0.8±0.2	2.0	3.0±1.1
Total lipid (µg P/mg protein)	0.95	3.58	11.18		4.17
Recovery from TLC	94.5±3.5				

The only lysophospholipids detected are LPC and LPE. Their concentration is very high in the zymogen granules, intermediate in mitochondria and very low in microsomes and whole homogenate. In the latter two the amount of lysophospholipids is not above the range, commonly found in several other tissues. Incubation of a whole homogenate for 60 min at $0^{o}C$ does not lead to a marked increase in lysophospholipid content of the homogenate (Table XII).

We have first investigated whether the high amounts of lysophospholipids in the zymogen granules could originate from enzymatic action during fractionation, and if so, which enzyme is responsible.

Phospholipase and lysophospholipase activities in subcellular fractions

The two types of phospholipase activity, which could be responsible for the high lysophospholipid contents, are determined in homogenate and subcellular fractions. The first is phospholipase A_2, known to be present in porcine pancreas as an inactive proenzyme, which can be activated by trypsin or by autolysis (De Haas et al., 1968b). The active form hydrolyses the 2-acyl bond of phospholipids, leading to the formation of 1-acyl lysophospholipids (De Haas et al., 1968a), which can be further broken down by lysophospholipase present in bovine pancreas (Shapiro, 1953). The second type is phospholipase A_1 which catalyses the formation of 2-acyl lysophospholipids (Van den Bosch et al., 1965). Presumably part of this activity may be attributed to pancreatic lipase which can split not only triglycerides but also the 1-acyl bond of phospholipids (Slotboom et al., 1970).

We have determined the activities of phospholipase and

TABLE XII

PHOSPHOLIPID COMPOSITION OF PIG PANCREATIC HOMOGENATE BEFORE AND AFTER INCUBATION AT $0^{o}C$.

Fresh homogenate is extracted immediately or after 60 min incubation at $0^{o}C$. Phospholipid composition of the extract is determined as described in section 7.2. Values, given as percentages of lipid-P, represent averages with S.E. of the mean. The number of experiments is given in parentheses at the top of the column.

Phospholipid	before incubation (4)	after incubation (4)
Origin	0.2 ± 0.2	0.3 ± 0.2
LPC	0.3 ± 0.3	1.6 ± 0.7
PC	49.6 ± 1.7	46.6 ± 1.2
LPE	0.4 ± 0.3	1.6 ± 0.6
PE	27.0 ± 0.8	26.5 ± 0.4
Sphingomyelin	4.1 ± 0.6	4.5 ± 0.2
PI + PA	8.8 ± 0.3	9.4 ± 0.1
PS	4.0 ± 0.7	4.6 ± 0.4
Cardiolipin	2.5 ± 0.9	2.2 ± 0.4
Front	3.0 ± 1.1	2.6 ± 0.6

lysophospholipase in the various subcellular fractions to see whether their distribution is correlated with that of the lysophospholipids. Phospholipase activities are determined before and after autolytic activation (Table XIII). The phospholipase activity does not increase linearly with the protein concentration of the homogenate or subcellular fractions. Under the conditions of our assay, both before and after activation, there is much more hydrolysis of PE in the 2-acyl position than in the 1-acyl position. It must be kept in mind, however, that during the assay at 37°C already some activation of phospholipase A_2 may have occurred, which may also explain at least partly the nonlinear characteristics of the enzyme activity. Finally, phospholipase A_2 activity is highest in zymogen granules, especially after activation. The considerable activity present in the mitochondrial fraction may be due, at least in part, to contamination of this fraction with zymogen granules. Lysophospholipase is even more specifically localized in zymogen granules than phospholipase. The data in the table indicate that both before and after activation phospholipase activity far exceeds that of lysophospholipase.

Experiments with radioactive phospholipids

The possibility that the large amounts of lysophospholipids are formed during fractionation of the pancreas, has been checked by adding radioactive PC to the homogenate before fractionation. The PC is partly labeled in the fatty acid at the 1-acyl position (^3H) and partly in the fatty acid in the 2-acyl position (^{14}C). Poor results are obtained, as only very little of the originally added radioactivity is associated with the sedimentable fractions after fraction-

TABLE XIII

DISTRIBUTION OF PHOSPHOLIPASE A_1 AND A_2 ACTIVITIES OVER SUBCELLULAR FRACTIONS

Phospholipase A_1 and A_2 activities are determined as described in section 7.2., in the fractions of the same experiments as in Table X. Values between parentheses are obtained with fractions, which are kept at room temperature for 16 hours.

		Phospholipase A_1 and A_2		Lysophospholipase
Fraction	mg protein	nmol ^{3}H-fatty acid released/ 15 min/mg/protein	nmol ^{14}C-fatty acid released/ 15 min/mg/protein	nmol ^{14}C fatty acid released/ 15 min/mg protein
Homogenate	0.092	22.8 (38.0)	224 (273)	23.5
	0.184	36.0	143	
Zymogen granules	0.090	34.4 (67.7)	238 (1177)	72.5
	0.180	70.0	245	
Mitochondria	0.104	15.4 (26.9)	151 (716)	17.5
	0.208	26.8	103	
Microsomes	0.214	18.2 (25.9)	57 (47)	10.5
	0.428	28.5	44	
PMS	0.083	6.0 (13.3)	144 (212)	28.5
	0.166	10.2	137	

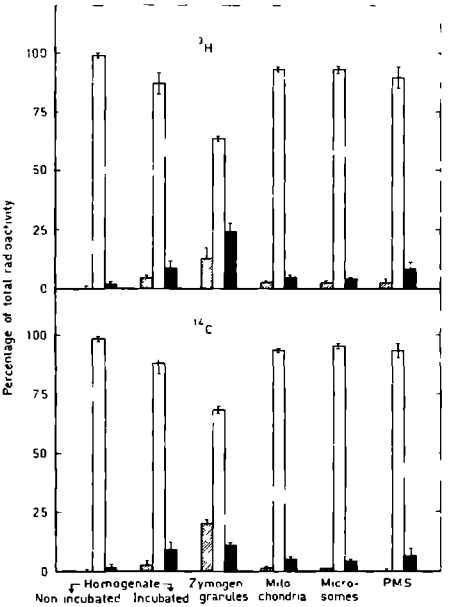

Fig. 34. Hydrolysis of added radioactive PC by homogenate and subcellular fractions at 0°C. ^{3}H-PC (150 nmol, 5,000 cpm) and ^{14}C-PC (3.3 nmol, 10,000 cpm), suspended in 0.25 ml 0.3 M sucrose, are mixed with 0.25 ml of the different subcellular fractions, suspended in 0.3 M sucrose. After incubation for 1 hour at 0°C, lipids are extracted. PC, LPC and fatty acids are separated according to Skipski et al. (1964) and radioactivity in the various spots is counted after combustion. Values are given as percentages of total recovered radioactivity and are averages of two separate experiments with standard errors of the mean. Hatched bars: LPC; white bars: PC; black bars: fatty acid. Values are corrected for impurities, present in the labelled PC. Average amounts of protein in 0.25 ml of the homogenate and subcellular fractions: homogenate: 12.4 mg; zymogen granules: 2.8 mg; mitochondria: 4.8 mg; microsomes: 13.0 mg; PMS: 5.7 mg.

ation, most of it apparently being lost during the washing procedures. Therefore, we have first isolated the various fractions, then added radioactive PC to them and incubated them for 1 hour at $0^{\circ}C$, as described in section 7.2. Next the phospholipids are extracted, separated by TLC, and converted to CO_2 and H_2O by combustion. The results of two experiments, shown in Fig. 34., indicate that a marked conversion of PC occurs in the zymogen granule fraction, but that little conversion occurs in other fractions. The products are free fatty acid as well as 1-acyl and 2-acyl LPC, the second being formed in slightly larger amount. These results suggest that the lysophospholipids in the zymogen granules are indeed formed during fractionation of the pancreas and that phospholipase A_2 as well as phospholipase A_1 or lipase are responsible for this hydrolysis at low temperature.

Fatty acid composition of phospholipids isolated from zymogen granules

Comparision of the fatty acid composition of the lysophospholipid and its parent phospholipid should indicate, which type(s) of lipolytic activity may be responsible for the enzymatic phospholipid degradation during isolation of the zymogen granules. Fatty acids are not randomly distributed in phospholipids: unsaturated fatty acids are preferentially located in the 2-acyl position, and saturated fatty acids in the 1-acyl position. Fatty acid analysis of PC, LPC, PE and LPE and of the total zymogen granule phospholipids has been executed (Table XIV). There is no marked difference in fatty acid composition of LPC and LPE on the one side and PC and PE on the other side, although in the

TABLE XIV

FATTY ACID COMPOSITION OF SOME PHOSPHOLIPIDS EXTRACTED FROM ZYMOGEN GRANULES.

Phospholipids are extracted from zymogen granules and thereafter separated by thin-layer chromatography. Fatty acids are determined by gas chromatography. Technical details are given in section 7.2. The results are presented as average percentage of the total amount of fatty acids with S.E. of the mean. The number of experiments is given between parentheses. In the phospholipid fractions only the 4 or 5 major fatty acids are determined, their sum being set at 100%.

Fatty acid[x]	Total granules (5)	LPC (7)	PC (6)	LPE (7)	PE (3)
12:0?	0.0±0.3				
14:0	1.9±0.6				
16.0	21.9±0.6	44.5±1.2	44.2±2.0	29.1±2.8	21.2±3.6
16.1	1.3±0.2				
17:0	0.2±0.1				
18·0	15.4±0.2	26.6±1.7	19.4±1.8	33.0±3.0	27.4±1.9
18:1	19.7±1.7	14.3±0.9	16.0±1.5	13.3±1.6	16.5±0.8
18·2	30.4±0.3	14.5±1.4	20.2±1.1	18.5±4.0	26.3±5.7
-	1.2±0.3				
-	0.3±0.3				
20:4	6.8±0.4			5.9±1.4	8.3±4.1

[x] Numbers in this column refer to chain length and number of double bonds, respectively.

lysophospholipids saturated fatty acids are slightly more predominant than in the corresponding phospholipids. This would suggest that if the lysophospholipids are the products of enzymatic breakdown, hydrolysis at both the 1-acyl and the 2-acyl position occurs, the latter position being slightly more vunerable.

Experiments with inhibitors of lipolytic enzymes

If the large amount of lysophospholipids found in zymogen granules is indeed due to enzymatic breakdown of phospholipids during tissue fractionation, inhibition of the responsible enzymes during fractionation should reduce the amounts of lysophospholipids in the zymogen granule fraction. Therefore, zymogen granules have been isolated in the presence of inhibitors, whereupon the phospholipid composition is determined. The following substances are used: Cd^{2+} (0.25 mM), which is a phospholipase A_2 inhibitor (De Haas et al., 1968a), and para-chloromercuribenzoate (0.1 mM) and paraoxon (0.25 mM), both of which are lipase inhibitors (Wills, 1960; Desnuelle et al., 1960). Pig pancreatic lipase is completely inhibited within 2 min by 0.25 mM paraoxon under the assay conditions. The results of the experiments (Table XV) indicate that of the lipase inhibitors, at least in the concentrations used, only paraoxon causes a slight decrease in the lysophospholipid content of the zymogen granules. However, it cannot be concluded that lipase plays no important role in the formation of lysophospholipids, since the concentrations of the inhibitors may have been too low for inhibition under the conditions of the fractionation. Higher concentrations cannot be used, because in that case no satisfactory isolation of zymogen granules is achieved.

TABLE XV

PHOSPHOLIPID COMPOSITION OF ZYMOGEN GRANULES AND THEIR MEMBRANES IN THE PRESENCE OF INHIBITORS OF LIPOLYTIC ENZYMES

Aliquots of zymogen granules or their membranes, isolated in the presence of inhibitors of lipolytic enzymes, are extracted according to Folch et al. (1957). Phospholipid composition of the extracts is determined as described in section 7.2. Values are given as percentage of lipid-P and represent averages. The number of experiments is given in parentheses at the top of the column.

Phospholipid	I Control (12)	II PCMB 0.1 mM (2)	III paraoxon 0.25 mM (2)	IV Cd^{2+} 0.25 mM (1)
Origin	0.2	0.3	0.5	0.7
LPC	12.8	11.7	9.2	5.2
PC	29.8	27.6	31.7	35.5
LPE	14.4	15.6	11.2	5.8
PE	18.1	14.7	18.8	22.0
Sphingomyelin	10.0	13.9	7.5	9.0
PI + PA	9.2	8.4	8.1	10.9
PS	0.1	-	-	-
Cardiolipin	2.9	1.7	5.7	3.7
Front	1.9	4.3	5.8	7.2

Table XV indicates that the presence of 0.25 mM Cd^{2+} during isolation reduces the amount of lysophospholipids by about 50%, suggesting that phospholipase A_2 plays a crucial role in the process of phospholipid breakdown. However, the zymogen granules isolated in this case are morphologically in poor shape.

7.4. DISCUSSION

In the first part of this chapter we report that zymogen granules, isolated at $0^{o}C$ from pig pancreas, contain an unusually high amount of LPC and LPE. A high content of LPC has previously been found in catecholamine containing secretory granules of adrenal medulla (Blaschko et al., 1967) and in pancreatic zymogen granules of ox (White and Hawthorne, 1970) and guinea pig (Meldolesi et al., 1971b). In ox pancreas this finding is attributed by the authors to the action of phospholipase A during fractionation at low temperature, while in the guinea pig lipase is held responsible.

However, some major differences between our findings and those of the above authors as well as some other observations still left some doubt in our minds as to these conclusions. First, in contrast to these authors, we find considerable amounts of LPE. Since they used the one-dimensional thin layer method of Skipski et al. (1964) for separation of phospholipids, they may have missed LPE, which almost coincides with PC in this system. Secondly,we do not find significant amounts of lysophospholipids either in microsomes, whose isolation takes a considerably longer time than is the case for zymogen granules and mitochondria, and in tissue homogenate. In mitochondria of pig pancreas the

amounts of lysophospholipids are also considerable, but much less than in zymogen granules. This may be due to the contamination of this fraction by zymogen granules; another possible explanation is given below. Thirdly, incubation of pancreatic homogenate at $0^{o}C$ does not lead to formation of lysophospholipids from endogenous lipids. Lastly, the stability of the isolated zymogen granules is remarkable, since the presence of such high amounts of lysophospholipids would be expected to destroy their structure.

The presence in an active form of enzymes, which can convert phospholipids to their lyso-derivatives, has been determined in the isolated fractions. Both phospholipase A_2 and some phospholipase A_1 activity seem to be present under our assay conditions and are predominantly located in the zymogen granules, as would be expected for secretory enzymes. The considerable activities in the mitochondrial fraction may be due to contamination with zymogen granules. Autolytic activation causes a large increase in phospholipase activity; this increase is relatively highest in those fractions, which have the highest activity before activation. Because of the non-linear characteristics of both enzyme activities and the possibility that phospholipase A_2 may already have been partly activated during the assay procedure, the absolute values cannot give a reliable quantitative picture of the relative activities of both enzymes but merely a qualitative indication. Lysophospholipase shows nearly the same distribution pattern as phospholipase and amylase, suggesting that it also is a secretory enzyme. Under assay conditions, i.e. at saturating substrate concentrations, lysophospholipase has a much smaller activity than phospholipase A_2.

The experiments, in which isolated subcellular fractions are incubated at $0^{o}C$ with radioactive PC, further support the idea that the lysophospholipids are formed enzymatically during fractionation. Zymogen granules hydrolyse PC, labeled in the 2-acyl fatty acid with ^{14}C, as well as PC, labeled with ^{3}H in the 1-acyl fatty acid, to a considerable extent, slightly more at the 1-acyl site. In other fractions little or no breakdown takes place. Homogenates also show some hydrolysis, which is in contrast to the experiments described in Table XII. However, in the latter experiments only breakdown of endogenous phospholipids has been measured, while the information here refers to exogenous phospholipids. Generally, however, these results agree with those of phospholipid analysis of the subcellular fractions, insofar as only in zymogen granules large amounts of lysophospholipids are found. Another conclusion, which can be drawn, is that enzymatic hydrolysis of the 1-acyl bond as well as the 2-acyl bond takes place, which means that phospholipase A_2 and lipase or phospholipase A_1 are probably the responsible enzymes. This conclusion is strengthened by the results of the analysis of the fatty acid composition of the phospholipids of the zymogen granules. LPC and LPE and their parent phospholipids do not show much difference in fatty acid composition, the parent compound having a slightly lower content of saturated fatty acids. Since saturated fatty acids are preferentially found in the 1-acyl position and unsaturated ones in the 2-acyl position, the lysophospholipids must have been formed by the action of phospholipase A_2 and lipase. This implies that these enzymes have a considerable activity at $0^{o}C$.

The fact that the lysophospholipids are found in high

concentration in isolated zymogen granules, less in mitochondria, and not in microsomes or in whole homogenate, even after incubation, may be due to two reasons. First, the lipolytic enzymes are most concentrated in zymogen granules and to a lesser extent in mitochondria. Secondly, the homogenate and the microsomal fraction may contain inhibitory substances like trypsin inhibitor, which prevent activation of phospholipase A_2, and thus the breakdown of phospholipids. These substances may be washed out of the mitochondria during the purification procedure.

There are two differences between the enzyme assays carried out at 37°C (Table XIII) and the incubation experiments at 0°C (Fig. 34 and Table XIV). In the first place the enzyme assays suggest the preferential action of phospholipase A_2, while the incubation experiments suggest that both enzymes are equally responsible for the breakdown. This may either be due to differences in temperature dependence of the enzymes or to differences in substrate saturation or the presence of cofactors. Phospholipase A_2, which has to be activated by removal of a hexapeptide from its zymogen, may be relatively inactive at low temperatures. Secondly, there is the fact that the phospholipid splitting enzymes seem to occur in all fractions (Table XIII) but at 0°C are almost only active in the zymogen granules and to a minor degree in mitochondria.

Although we have thus shown that the presence of lysophospholipids in isolated zymogen granules can be explained by enzymatic conversion of phospholipids during tissue fractionation, we cannot completely rule out the possibility that in vivo granular lysophospholipids could play a role in the extrusion process. If they do play such

a role, one would expect a rapid formation and conversion of these substances in the zymogen granules in vivo. The rapid formation must be assumed in view of the considerable activity of lysophospholipid forming enzymes at $0^{o}C$. Whether the resulting lysophospholipids can also be rapidly converted, either by further degradation or by reacylation, is not shown by our experiments.

CHAPTER VIII

SECRETION OF CALCIUM AND MAGNESIUM BY THE ISOLATED RABBIT PANCREAS

8.1. INTRODUCTION

In previous chapters experiments have been described, which were devised to investigate, whether cyclic AMP is the "second messenger" in the hormone stimulation of the exocrine secretion by the pancreas. Although several of our findings favour such a view, no unequivocal answer to this problem could be given. Hence it seems reasonable to consider the possibility that other factors may be important or even rate-limiting in the stimulus-secretion process.

In the past few years a possible involvement of calcium ions in coupling mechanisms has received much attention. In many secretory systems there exists ample evidence for such a role (see Rubin, 1970). With regard to the process of exocrine pancreatic secretion some of the arguments pleading for an involvement of Ca^{2+} have already been reviewed in section 1.4., e.g. the requirements of extracellular calcium for the secretory response of the pancreas to pancreozymin and acetylcholine and the depolarizing effect of these compounds on the membrane potential of the acinar cell.

An increase in enzyme secretion by the dog pancreas in vivo is parallelled by an increased secretion of Ca^{2+} (Zimmerman et al., 1967; Goebell et al., 1972). The same observation has been made for the perfused cat pancreas

(Argent et al., 1973). In all cases it is concluded that at least part of the secreted Ca^{2+} is bound to or associated with the secreted enzymes.

Since the pattern of Ca^{2+} secretion in relation to enzyme secretion may yield information about the importance of Ca^{2+} movements in the secretion process, we have tested in the isolated rabbit pancreas the secretion of Ca^{2+} into the juice, both in the resting and in the stimulated organ. The pattern of Mg^{2+} secretion has also been studied in order to see whether there are differences in secretory behaviour between the two bivalent cations. The results of these experiments are described in this chapter and the physiological significance of the findings is discussed.

8.2. METHODS

Preparation of the isolated rabbit pancreas

The experimental procedure with the isolated pancreas is as described in section 2.2. The only difference is that in this group of experiments the NaCl concentration in the bathing medium has been lowered somewhat, the final Na^{+} concentration being 143.5 mM and the final Cl^{-} concentration 130.7 mM. This solution is nearer to isotonicity than the solution used in Chapter II.

Ca^{2+} and Mg^{2+} determinations

Ca^{2+} and Mg^{2+} concentrations in the fluid are measured in a double beam atomic absorption flame photometer (Perkin Elmer, model 403). The juice is diluted with 50 volumes of a solution of EDTA in water (0.37 mM = 125 mg/l). EDTA is added to prevent interference by phosphate present in the

TABLE XVI

BASAL SECRETION OF CALCIUM, MAGNESIUM AND PROTEIN

Protein, Ca^{2+} and Mg^{2+} concentrations are measured in the secreted fluid, which is collected in hourly periods. Averages are given with the S.E. of the mean for 5 experiments. Ca^{2+} and Mg^{2+} concentrations in the bathing medium are 2.5 and 1.2 mM.

number hourly period	flow µl/hr	protein mg/hr	Ca^{2+} µmol/hr	$[Ca^{2+}]$ mM	Mg^{2+} µmol/hr	$[Mg^{2+}]$ mM
1	287±104	4.23±1.24	0.62±0.17	2.28±0.24	0.18±0.05	0.71±0.14
2	378± 78	1.25±0.27	0.51±0.10	1.42±0.17	0.14±0.03	0.40±0.05
3	397± 74	0.95±0.25	0.50±0.11	1.22±0.07	0.15±0.04	0.39±0.05
4	342± 81	0.67±0.19	0.42±0.11	1.21±0.08	0.14±0.03	0.42±0.05
5	254± 78	0.51±0.13	0.32±0.10	1.22±0.07	0.11±0.03	0.44±0.04

fluid. Standard solutions of Ca^{2+} (6 - 30 µM) and Mg^{2+} (8 - 40 µM) in 0,37 mM EDTA are used to obtain calibration curves, which are linear in this concentration range.

Protein determination

Protein is determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

8.3. RESULTS

Basal Ca^{2+} and Mg^{2+} secretion

Table XVI shows the basal secretion of fluid, protein, Ca^{2+} and Mg^{2+} by the isolated rabbit pancreas. Secretion of fluid and protein follows the pattern as already described in section 2.3. Ca^{2+} and Mg^{2+} concentrations are highest in the first hour after mounting, decrease considerably during the second hour and reach a relatively constant level thereafter. The amounts of these ions secreted are also highest in the first hour, but the decline after the first hour is much less than for the concentrations. In view of the relatively constant flow and Ca^{2+} and Mg^{2+} secretion during the third and fourth hour, the effects of various stimulants on the bivalent cation secretion have been tested by adding the stimulants to the bathing medium at the end of the third hour of incubation.

Effect of carbachol and high K^{+} concentrations on Ca^{2+} and Mg^{2+} secretion

Table XVII shows the effect of 10^{-5}M carbachol on the secretion of protein, Ca^{2+} and Mg^{2+}. A three to fivefold rise in the concentrations of both cations is observed, but

TABLE XVII

EFFECT OF CARBACHOL ON THE SECRETION OF CALCIUM, MAGNESIUM AND PROTEIN

Protein, Ca^{2+} and Mg^{2+} concentrations are measured in the secreted fluid, which is collected in hourly periods. Carbachol (10^{-5}M) is added at the end of the third hour of incubation. Averages of 3 experiments are given with the S.E. of the mean. Ca^{2+} and Mg^{2+} concentrations in the bathing medium are 2.5 and 1.2 mM.

number hourly period	flow µl/hr	protein mg/hr	Ca^{2+} µmol/hr	$[Ca^{2+}]$ mM	Mg^{2+} µmol/hr	$[Mg^{2+}]$ mM
1	305± 57	4.84±1.42	0.40±0.05	1.46±0.04	0.18±0.04	0.65±0.25
2	418± 59	1.36±0.50	0.29±0.04	0.70±0.21	0.14±0.02	0.34±0.05
3	527± 58	1.04±0.03	0.34±0.06	0.65±0.12	0.16±0.02	0.30±0.05
4	470± 59	14.60±2.86	1.02±0.13	2.19±0.16	0.69±0.11	1.46±0.02
5	409±109	3.61±1.03	0.66±0.16	1.16±0.10	0.40±0.12	0.97±0.04

the concentrations never exceed those in the bathing medium. The total amount of both cations secreted per hour also increases (200 and 331%), although relatively less than the amount of protein (1300%). A representative experiment is shown in Fig. 35.

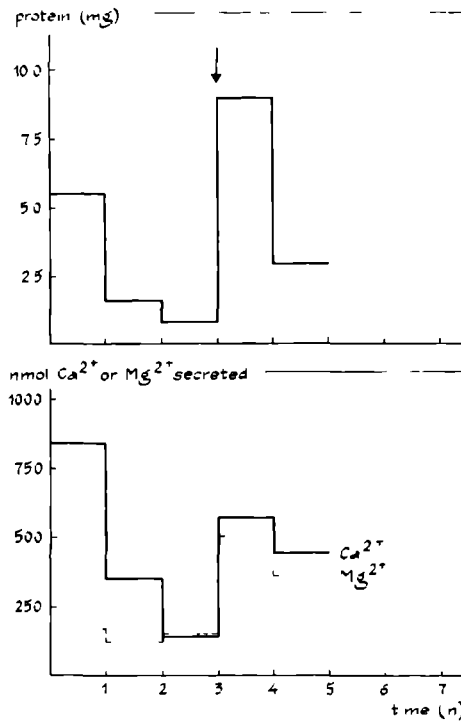

Fig. 35 The secretion of Ca^{2+}, Mg^{2+} and protein by the isolated rabbit pancreas. At the end of the third hour 10^{-5}M carbachol is added. Other conditions are as described in section 8.2.

TABLE XVIII

EFFECT OF A HIGH POTASSIUM CONCENTRATION IN THE BATHING MEDIUM ON THE SECRETION OF CALCIUM, MAGNESIUM AND PROTEIN

Protein, Ca^{2+} and Mg^{2+} concentrations are determined in the fluid, which is collected in hourly periods. At the end of the third hour the bathing medium is replaced by a similar medium, in which NaCl is partly replaced by an equivalent amount of KCl, the final K^{+} concentration being 75 mM. Averages of 5 experiments are given with the S.E. of the mean. Ca^{2+} and Mg^{2+} concentrations in the bathing medium are 2.5 and 1.2 mM.

number hourly period	flow µl/hr	protein mg/hr	Ca^{2+} µmol/hr	$[Ca^{2+}]$ mM	Mg^{2+} µmol/hr	$[Mg^{2+}]$ mM
1	303±36	3.96±0.41	0.49±0.08	1.67±0.28	0.17±0.02	0.59±0.07
2	451±28	2.26±0.58	0.58±0.04	1.30±0.16	0.20±0.02	0.46±0.07
3	429±28	1.23±0.36	0.57±0.08	1.34±0.17	0.21±0.03	0.52±0.09
4	249±27	4.76±2.11	0.51±0.07	2.04±0.19	0.24±0.03	0.98±0.06
5	271±38	2.59±1.08	0.57±0.12	1.97±0.28	0.29±0.05	1.10±0.08

Since high external potassium has been found to stimulate protein secretion by the isolated rabbit pancreas, probably by releasing acetylcholine from intrapancreatic stores (see section 2.4.), the effect of high concentrations of this ion (75 mM) in the bathing medium has also been studied (Table XVIII). While protein secretion is stimulated, the total amounts of Ca^{2+} and Mg^{2+} secreted are not enlarged. However, the concentration of Ca^{2+} increases to about the same concentration as in the presence of 10^{-5}M carbachol. For Mg^{2+} a smaller increase is observed. The concentrations of the bivalent cations remain elevated during the second hour after raising the K^{+} concentration (fifth hourly period).

Effect of tetracaine on the carbachol stimulated Ca^{2+} and Mg^{2+} secretion

Since local anesthetics, such as tetracaine, are known to inhibit Ca^{2+} movements across membranes, the effect of tetracaine on the carbachol stimulated secretion of Ca^{2+}, Mg^{2+} and protein has been examined. In these experiments 10^{-4}M tetracaine is added at the end of the third hour, together with 10^{-5}M carbachol (Table XIX). The flow rate decreases more (41%) than with carbachol alone (11%), but the protein secretion is still stimulated (3-fold vs. 14-fold with carbachol alone). The total amounts of Ca^{2+} and Mg^{2+} secreted per hour rise significantly, but the concentrations of Ca^{2+} and Mg^{2+} increase to approximately the same values as in the bathing medium, as is also the case in the presence of a high external K^{+} concentration.

TABLE XIX

EFFECT OF TETRACAINE ON THE SECRETION OF CALCIUM, MAGNESIUM AND PROTEIN, STIMULATED BY CARBACHOL

Protein, Ca^{2+} and Mg^{2+} concentrations are measured in the fluid, which is collected in hourly periods. At the end of the third hour 10^{-5}M carbachol is added together with 10^{-4}M tetracaine. Averages of 3 experiments are given with the S.E. of the mean. Ca^{2+} and Mg^{2+} concentrations in the bathing medium are 2.5 and 1.2 mM.

number hourly period	flow µl/hr	protein mg/hr	Ca^{2+} µmol/hr	$[Ca^{2+}]$ mM	Mg^{2+} µmol/hr	$[Mg^{2+}]$ mM
1	312±41	5.80±1.23	0.65±0.10	2.09±0.20	0.17±0.02	0.54±0.05
2	406±80	2.21±0.83	0.55±0.14	1.35±0.06	0.16±0.03	0.40±0.10
3	410±87	2.18±0.93	0.54±0.12	1.31±0.04	0.18±0.03	0.47±0.01
4	244±78	8.56±4.08	0.68±0.26	2.57±0.40	0.30±0.09	1.17±0.11
5	249±32	4.40±0.82	0.53±0.08	2.12±0.10	0.24±0.02	0.98±0.08

8.4. DISCUSSION

The calcium concentration in the fluid secreted by the isolated, non-stimulated pancreas runs parallel to the protein concentration, being relatively high during the first hour after mounting, thereafter declining and becoming relatively constant after the second hour. The calcium concentration then reached averages 1 mM, which is far less than the calcium concentration in the bathing medium (2.5 mM). This seems to be in contrast with findings in the dog, where the fluid secreted by the non-stimulated organ in vivo contains Ca^{2+} in concentrations only slightly lower than (Zimmerman et al., 1967) or equal to the serum concentration (Goebell et al., 1972). However, in both cases, when fluid secretion is stimulated by intravenous injection of secretin, the Ca^{2+} concentrations in the fluid decrease to values below 1 mM. For the perfused cat pancreas Argent et al. (1973) find a mean Ca^{2+} concentration of 0.31 mM in the secreted fluid when the perfusate (1.25 mM Ca^{2+}) contains submaximally stimulating concentrations of secretin. Taking into account that the isolated rabbit pancreas with respect to fluid secretion behaves as if almost maximally stimulated (Rothman, 1964; Ridderstap, 1969), our results are not incompatible with those of other authors.

In response to the secretory stimulant carbachol the Ca^{2+} concentration in the fluid secreted by the isolated rabbit pancreas, as well as the amount of this ion secreted per hour, increase in parallel with the secretion of protein. The observed concentrations do not exceed the concentration in the bathing medium. This confirms the observations of Zimmerman et al. (1967) and Goebell et al. (1972) for the in vivo dog pancreas, and also of Argent et al. (1973) for

TABLE XX

RELATION BETWEEN AMOUNTS OF CALCIUM, MAGNESIUM AND PROTEIN, SECRETED BY THE ISOLATED RABBIT PANCREAS UNDER DIFFERENT CONDITIONS

The values presented in this table are derived from the experiments, shown in Tables XVI, XVII, XVIII and XIX. They are given as mean values.

$$I = \frac{\mu mol\ Ca^{2+}}{mg\ protein}$$

$$II = \frac{\mu mol\ Mg^{2+}}{mg\ protein}$$

$$III = \frac{\mu mol\ Ca^{2+}}{\mu mol\ Mg^{2+}}$$

number hourly period	basal secretion			$+10^{-5}$M carbachol			$+10^{-5}$M carbachol $+10^{-4}$M tetracaine			+75 mM K^{+}		
	I	II	III	I	II	III	I	II	III	I	II	III
1	0.146	0.042	3.14	0.082	0.037	2.22	0.112	0.029	3.82	0.123	0.042	2.88
2	0.408	0.112	3.64	0.213	0.102	2.07	0.248	0.072	3.43	0.256	0.088	2.90
3	0.526	0.157	3.33	0.326	0.153	2.12	0.247	0.082	3.00	0.463	0.170	2.71
4	0.626	0.208	3.00	0.069	0.047	1.47	0.035	0.035	2.26	0.107	0.050	2.12
5	0.627	0.215	2.90	0.182	0.110	1.65	0.054	0.054	2.20	0.220	0.111	1.96

the perfused cat pancreas. Although elevation of the potassium concentration in the bathing medium enhances the protein secretion, the amount of Ca^{2+} secreted per hour does not increase, although the concentration approaches that in the bathing medium. The same is true when 10^{-4}M tetracaine is added together with carbachol, although a small stimulation of the Ca^{2+} secretion (26%) is observed.

For Mg^{2+} a similar pattern is observed under all these conditions, although the concentrations of this ion in the fluid are always approximately half of the calcium concentration.

In Table XX the results from the experiments in Tables XVI - XIX are summarized. The Ca^{2+}/protein ratio varies from 0.035 to 0.627 µmol/mg. The Mg^{2+}/protein ratio also varies considerably (0.029 to 0.215 µmol/mg). The variability of these ratio's suggests that Ca^{2+} and Mg^{2+} are not exclusively secreted in a protein-bound form.

It could be that calcium and magnesium in part accompany the protein secretion and in part are constant, basal components of the secreted fluid. This possibility can be tested by plotting the Ca^{2+} and Mg^{2+} amounts as a function of the protein concentration in the secreted fluid and determining whether the points can be fitted to equations of the following form:

$$Y = A + BX,$$

where Y represents the Ca^{2+} or Mg^{2+} content in the secreted fluid (in µmol), A the constant fraction (in µmol) and BX the fraction associated with the enzymes (B in µmol/mg protein, X in mg protein). The regression line for Ca^{2+} vs. protein for 18 diverse experiments, in which 5 hourly periods are analyzed, is shown in Fig. 36. A high corre-

lation coefficient is found: r = 0.715;(P < 0.001; n = 90). The value for B is 0.046 µmol/mg protein and for A 0.38 µmol. The regression line has a relatively low slope, while A is relatively high. This means that even at high rates of protein secretion (up to 9 mg/hour) the major part of the Ca^{2+} is not secreted in association with the enzymes. These results contradict those of Zimmerman et al. (1967) for the dog pancreas in vivo, where all of the secreted Ca^{2+} was found associated with the enzymes. However, they are in agreement with those of Goebell et al. (1972), who also studied the dog pancreas in vivo, and those of Argent et al. (1973) for the isolated perfused cat pancreas.

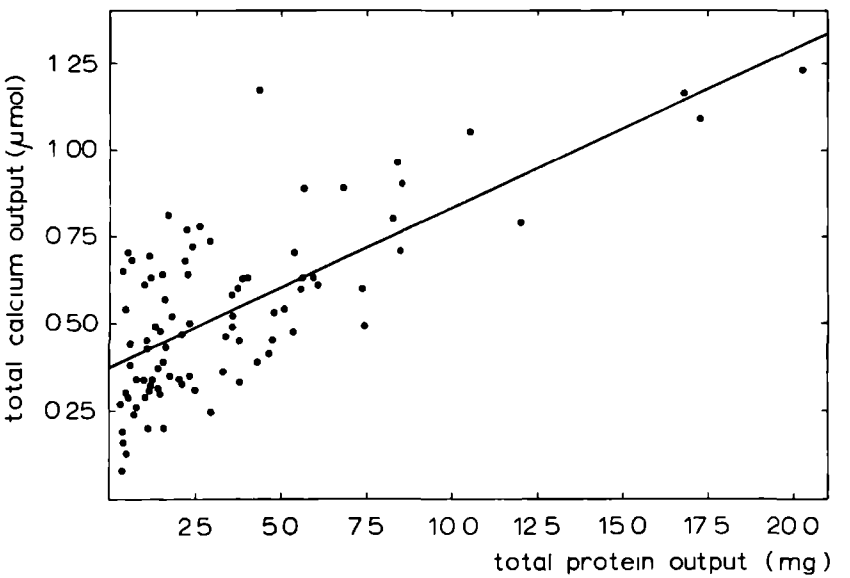

Fig. 36. The secretion of Ca^{2+} and protein by the isolated rabbit pancreas. The points represent a total of 90 observations from 18 experiments, in which the pancreas is either non-stimulated or stimulated in one of the manners described in section 8.3. The solid line is the calculated regression line.

Both groups of authors find a similar relationship between Ca^{2+} and enzyme secretion as we do.

If we take out from these points the relatively high protein values (> 2 mg/hour), approximately the same regression line is calculated ($r = 0.713$; $P < 0.001$; $A = 0.40$ µmol; $B = 0.042$ µmol/mg protein; $n = 49$). When the points at low protein values (> 1 mg/hour) are taken, no correlation between Ca^{2+} and protein is found ($r = -0.04$; $P > 0.10$; $n = 19$). This means that the low protein values do barely contribute to the shape of the regression line.

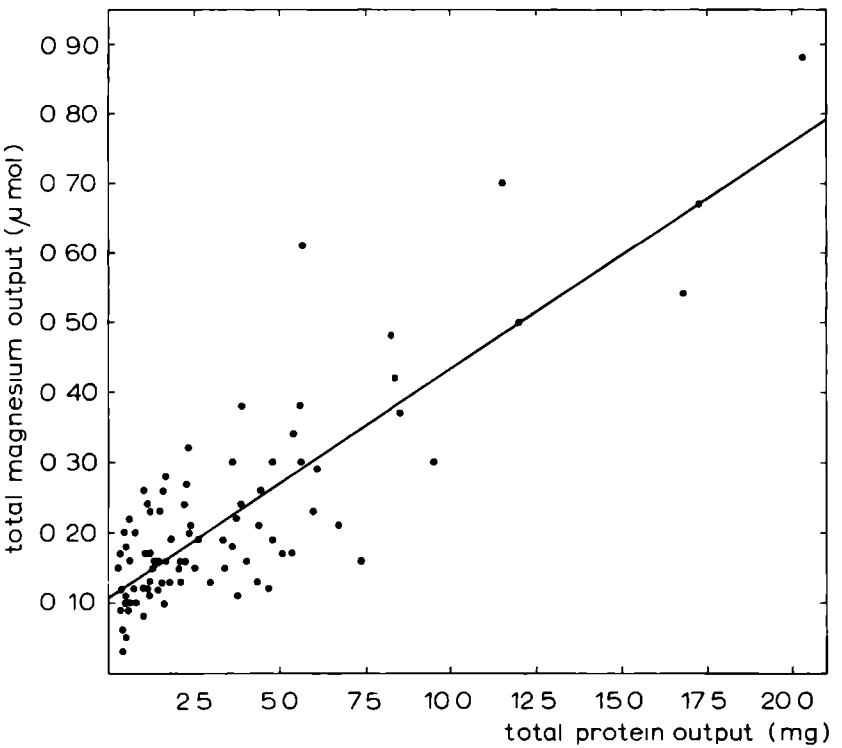

Fig. 37. The secretion of Mg^{2+} and protein by the isolated rabbit pancreas. The points are derived from the same observations as in Fig. 36. The solid line is the calculated regression line.

For the relation between Mg^{2+} and protein secretion another regression line can be calculated. This line has an even higher correlation coefficient ($r = 0.836$, $P < 0.001$; $A = 0.11$ µmol; $B = 0.033$ µmol/mg protein; $n = 49$; see Fig 37.).

Despite these high correlation coefficients we may not draw the conclusion that the protein independent part of the calcium and magnesium secretion is also physiologically constant. A possible relation of the calcium and magnesium concentration with the flow rate, as discussed before, should be examined. This can be done by determining the fit of the measured values to the equation:

$$Y = A + BX + CZ,$$

where C represents the flow dependent Ca^{2+} component (in µmol/µl) and Z the flow rate in µl/hour. The other symbols have the same meaning as in the previous equation. The values for A, B and C have been calculated with help of the method of least squares. They have to be relatively constant for all experiments and they must also be physiologically acceptable. However, this only proves to be the case for the experiments under basal conditions but not for the experiments under stimulated conditions, for which highly different values are found. It seems,therefore,that a secretion model for Ca^{2+}, as represented by the above equation, must be rejected. As to this, however, a definite conclusion can only be made after obtaining more measurements.

Furthermore, there are some indications that even such an approach will not be accurate enough to describe the physiological situation. Preliminary experiments by Drs. V.V.A.M. Schreurs in our laboratory with ^{45}Ca in the bathing medium indicate the presence of a distinct calcium flux dependent on stimulation by a secretagogue. This could be

due to an increased calcium uptake at the serosal side of the acinar cell in response to a secretory stimulus. This is in agreement with the situation observed in a number of other secretory processes. For many of these secretory processes entrance of calcium into the cell seems to be necessary for the initiation of secretion. Evidence leading to this conclusion is that the influx of calcium is stimulated by secretory stimulants and also that the extracellular presence of Ca^{2+} is necessary for stimulation of secretion (see Simpson, 1968; Rubin, 1970). Recent publications also show that externally applied calcium ionophores increase the secretion of histamine by mast cells (Foreman et al., 1973). Injection of Ca^{2+}, but not of Mg^{2+}, into these cells stimulates secretion of histamine (Kanno et al., 1973). An increased influx of ^{45}Ca in rat pancreatic fragments on stimulation with pancreozymin has been observed (Case and Clausen, 1973). Calcium-free media or perfusion fluids abolish the secretory response to secretagogues of pigeon pancreas slices (Hokin, 1966), rat pancreatic fragments (Robberecht and Christophe, 1971; Heisler et al., 1972; Kanno, 1972; Case and Clausen, 1973), guinea pig pancreas slices (Benz et al., 1972) and the isolated cat pancreas (Argent et al., 1973). However, basal secretion is only slowly affected. Also a reduction of the Ca^{2+} concentration in the medium from 2.5 to 0.1 mM has no effect on the stimulation of amylase secretion by pancreozymin in rat pancreatic fragments (Heisler et al., 1972; Case and Clausen, 1973) as well as on the efflux of ^{45}Ca from preloaded rat pancreatic fragments (Case and Clausen, 1973). These observations seem to reflect an increased permeability of the serosal membrane to Ca^{2+} and prove the necessity of the presence of some extracellular Ca^{2+} for the initiation

of the secretion process. The primary event would be a depolarization of the serosal membrane, which is known to occur in pancreatic acinar cells after stimulation with a secretagogue (Dean and Matthews, 1972, Petersen and Matthews, 1972). This would be accompanied by an increased Na^+ influx. The entrance of Na^+ triggers release of calcium from intracellular pools, e.g. endoplasmatic reticulum and mitochondria, which are known in other cell types to contain much higher concentrations of this ion than cytoplasma

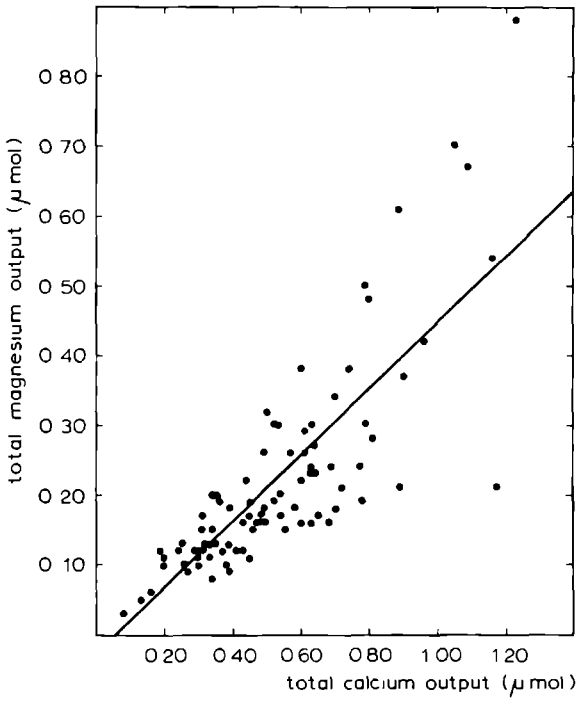

Fig. 38. The secretion of Ca^{2+} and Mg^{2+} by the isolated rabbit pancreas. The points are derived from the same observations (n = 90) as in Fig. 36. The solid line is the calculated regression line.

(Carvalho, 1968; Feinstein and Schramm, 1969; Lehninger, 1970). In favour of this assumption is the finding that replacement of sodium in the bathing medium by lithium depresses the effect of pancreozymin on amylase release and ^{45}Ca efflux from preloaded rat pancreatic fragments (Case and Clausen, 1973). The possibility that the entrance of Na^+ is the primary event in pancreatic stimulus-secretion coupling has been discussed before (Case, 1973a; 1973b).

In Table XX the Ca^{2+}/Mg^{2+} ratio obtained in the various experiments is also shown. This ratio is relatively constant, varying only from 1.47 to 3.82. This is further shown by plotting Ca^{2+} secretion as a function of Mg^{2+} secretion and calculating the regression line. A high correlation coefficient is found: $r = 0.801$ ($P < 0.001$; $n = 90$; see Fig. 38.). This regression line nearly goes through the origin, while the slope is 0.48 µmol Mg^{2+}/µmol Ca^{2+}, which is exactly the same value as the ratio of the Mg^{2+} and Ca^{2+} concentrations in the bathing medium (1.2 and 2.5 mM, respectively). This means that there is a completely parallel secretion of both bivalent cations. This is a puzzling phenomenon, because pancreatic enzyme secretion is not dependent on the presence of extracellular Mg^{2+} (Argent et al., 1973). There is even a report that enzyme secretion may be depressed by its presence (Robberecht and Christophe, 1971) and in excitatory tissues Mg^{2+} is found to antagonize Ca^{2+} (see Simpson, 1968). Furthermore, the intracellular Mg^{2+} concentration is generally much higher than the Ca^{2+} concentration and may reach values as high as 20 mM (see White et al., 1968). We can offer no reasonable explanation for this phenomenon. One possibility, which should be mentioned, is that a ductular exchange mechanism between Ca^{2+} and Mg^{2+} exists.

The starting point for the investigations reported in this thesis has been the hypothesis of Ridderstap and Bonting (1969c) on the stimulus-secretion coupling in pancreatic enzyme secretion. Briefly stated this hypothesis is as follows: a. pancreozymin activates adenylate cyclase in the serosal acinar cell membrane; b. the resulting cyclic AMP stimulates a phospholipase in the zymogen granules and the lysophospholipids formed by the activated enzyme induce fusion of zymogen granules and luminal acinar cell membranes. The mechanism of action of acetylcholine, which causes essentially the same events in the acinar cell as pancreozymin does, has not been discussed by the authors.

The experiments to prove an intermediate role of the cyclic nucleotide (part a of the hypothesis) consist of three different types.

First we have tried to establish in the isolated rabbit pancreas, whether externally added cyclic AMP (1 mM) is able to mimick the effects of pancreozymin, as was formerly shown by Ridderstap and Bonting (1969c). We were not able to confirm these observations. On the other hand, the phosphodiesterase inhibitor papaverine does stimulate enzyme secretion. In section 2.4. we discuss these findings together with those of other authors, who report conflicting observations. It has been concluded that species differences may be responsible for the conflicting results.

Next, we have studied in rat pancreas the enzymes

which control the intracellular concentration of cyclic AMP, adenylate cyclase which synthesizes the nucleotide, and phosphodiesterase which catalyzes its hydrolysis. The first enzyme is present and is stimulated by both secretin and pancreozymin. Although a 100 times higher concentration of pancreozymin than of secretin is needed to cause half maximal stimulation, it could be shown that the effect of pancreozymin is not due to a contamination of the pancreozymin preparation by secretin. Acetylcholine has no effect on enzyme activity, although there are reports that in living pancreatic cells cyclic AMP levels may rise after stimulation with acetylcholine. These findings are discussed in section 4.4.

Thirdly, in chapters IV and V the presence and properties of phosphodiesterase in rat pancreas have been described. Two different activities have been observed, one with a high and the other with a low affinity for the substrate. The physiological significance of the first activity seems to be larger than that of the second. The presence of this enzyme does not necessarily prove that cyclic AMP plays a role in pancreatic secretory processes. The activity of the enzyme is not influenced by physiological secretagogues (secretin, pancreozymin and acetylcholine), but it is inhibited by other substances like theophylline and papaverine, which are known inhibitors of the enzyme in several other tissues.

These findings do not conclusively prove or disprove part a of the hypothesis: whether cyclic AMP has a mediating role in the hormonal stimulation of enzyme secretion. At the moment the evidence for such a role of cyclic AMP in fluid and electrolyte secretion is somewhat more con-

vincing (see sections 2.4. and 3.4.). It is possible that additional factors may play a role in the control of cyclic AMP levels in the acinar cell as well as in the response of the cell to an increased concentration of this nucleotide. Calcium ions may play such a role, as will be discussed below.

The second part (b) of the hypothesis is concerned with the question how an increased intracellular level of cyclic AMP could elicit the release of enzymes stored in the zymogen granules. In chapter VI we have studied the suggestion of Ridderstap and Bonting (1969c) that cyclic AMP may activate a phospholipase present in the pancreas. However, we could not demonstrate an effect of the nucleotide on pancreatic phospholipase and lipase activity, either when added during the assay or during a period of preincubation. In view of a possible role of a cyclic AMP activated protein kinase in such an activation (Kuo and Greengard, 1969), the effect of the nucleotide on lipase and phospholipase activities has also been studied in the presence of Mg^{2+} and ATP, which are known to be essential in the kinase phosphorylation step. However, also in this experiment cyclic AMP was without effect.

Next, we considered the possibility that cyclic AMP could directly influence membrane integrity. In chapter VI it is described that a relatively high concentration of the nucleotide (1 mM) induces a release of protein from isolated zymogen granules. The effect is rather specific (5'-AMP has no effect) but is also rather slow (30 - 60 min). Hence, we consider the physiological relevance of this effect doubtful.

In view of the suggestion in the hypothesis that lysophospholipids might induce membrane fusion during enzyme

secretion, we have studied the composition of the lipids of the granular membrane. In chapter VII it is reported that these membranes contain unusually high amounts of lysophosphatidylcholine and lysophosphatidylethanolamine. Although this has been shown to be an artefact, caused by lipolytic enzymes present in the pancreas, it has been discussed in section 7.4. that a role of this type of compounds in the final fusion process cannot be excluded at this moment.

In summary, then, we can state that part a of the hypothesis is correct, namely that pancreozymin stimulates adenylate cyclase activity in the pancreas, but that no confirmation has been obtained of part b, namely that cyclic AMP would stimulate (phospho)lipase activity. Furthermore, our inability to confirm an in vitro stimulation of enzyme secretion by exogenous cyclic AMP leaves some doubt as to the existence of a mediating role of the nucleotide in enzyme secretion.

Another factor, which may play a role in the stimulus-secretion coupling of pancreatic enzyme secretion, could be calcium. Chapter VIII describes that Ca^{2+} secretion by the isolated rabbit pancreas is stimulated by secretagogues. Partly this Ca^{2+} appears to be associated with the secreted enzymes. We must consider the possibility that an increase in cytoplasmatic Ca^{2+} concentration, by an influx from the serosal side and/or by a redistribution from subcellular pools, would be an essential step in the process of enzyme secretion.

In this context the general hypothesis for cell activation proposed by Rasmussen et al. (1972) must be discussed. In this model the first messenger, by its action on a receptor in the cell membrane, induces two simultaneous

events, an activation of adenylate cyclase leading to an increased intracellular concentration of cyclic AMP and to an influx of Ca^{2+} into the cell. The concentrations of both messengers are reciprocally controlled by each other. Cyclic AMP furthermore causes activation of protein kinase(s). The rise in intracellular Ca^{2+} concentration quenches the cyclic AMP response by inhibition of adenylate cyclase and activation of phosphodiesterase. On the other hand, Ca^{2+} is necessary for the activity of enzymes, which are phosphorylated by the cyclic AMP activated protein kinase(s). An additional function of Ca^{2+} would be to increase the permeability of cell membranes for monovalent cations. This model may explain the contradictory observation about the role of cyclic AMP in pancreatic enzyme secretion in different species, if we assume that the relative activities of cyclic AMP and Ca^{2+} as second messengers would differ for these species.

The model does not explain how acetylcholine causes enzyme secretion. Possibly this compound may increase the permeability of the serosal membrane to Ca^{2+}. One feature of the model we do not find confirmed in the pancreas, namely pancreatic phosphodiesterase is not activated or stimulated by Ca^{2+} (section 5.3.). Other points in this hypothesis also remain unclear, e.g. there is no evidence for the significance of cyclic AMP activated protein kinases in this process. Although the existence of such an enzyme in the pancreas has been proven (Kuo and Greengard, 1969), its nature and function are still unknown. Hence, it seems to early to decide whether Rasmussen's model is representing the physiological situation in the pancreas.

Further studies will have to concern the control of subcellular cyclic AMP levels as well as the Ca^{2+} concentra-

tion, if possible in living acinar cells. An identification of cyclic AMP activated protein kinase(s) will also be necessary. The elucidation of the process of membrane fusion will require a better understanding of the structural and chemical features of biological membranes and their interactions. This and related information appears to be necessary in order to obtain a full understanding of the process of stimulus-secretion coupling in pancreatic enzyme secretion.

De exocriene pancreas sekreteert een vloeistof, welke naast elektrolyten ook enzymen bevat, welke in de dunne darm een essentiele rol spelen in de vertering van het voedsel. Passage van voedsel door de maag en de darm geeft via nerveuze (de neurotransmitter acetylcholine) of neurohumorale weg (de hormonen secretine en pancreozymine) de prikkel tot sekretie aan de pancreas. Dit proefschrift beschrijft een studie van de processen, welke zich in de sekreterende cellen afspelen vanaf de stimulus door hormoon of neurotransmitter en de uiteindelijke sekretie. In het bijzonder wordt aandacht geschonken aan de rol hierin van het cyclisch AMP, waarvan in andere weefsels bekend is, dat het een intermediair is in de stimulus-sekretie koppeling.

Hoofdstuk I geeft een algemene inleiding over de processen, die zich in de cellen van de exocriene pancreas afspelen, alsmede van de faktoren, welke de sekretie reguleren. Tevens beschrijft dit hoofdstuk de hypothese van Ridderstap en Bonting (1969c), waarin gepostuleerd wordt, dat pancreozymine de sekretie van enzymen stimuleert via een aktivering van het enzym adenylaat cyclase in de celmembraan. Het hierdoor gevormde cyclisch AMP zou dan een phospholipase A stimuleren, welk enzym fosfolipiden afbreekt tot lysofosfolipiden. Deze laatste verbindingen zouden de samensmelting kunnen bewerkstelligen van de plasmamembranen met de membranen van de zg. zymogeengranula, in welke laatste subcellulaire strukturen de reeds gevormde spijsverteringsenzymen zijn opgeslagen, indien de pancreas niet gestimuleerd is. Een groot gedeelte van het onderzoek, beschreven in dit proefschrift, is uitgevoerd om

deze hypothese te toetsen.

Hoofdstuk II beschrijft experimenten met de geisoleerde konijnepancreas. De effekten van verschillende verbindingen, zoals theophylline en papaverine, cyclisch AMP zelf, en van een hoge kaliumconcentratie in de badvloeistof, op de vloeistof- en elektrolytsekretie en de enzymsekretie worden bestudeerd. Van deze verbindingen is bekend, dat in diverse andere weefsels de intracellulaire concentratie van cyclisch AMP erdoor verhoogd wordt. Papaverine, zowel als een hoge kaliumconcentratie in de badvloeistof, remmen de sekretie van vloeistof en elektrolyten. Papaverine en kalium, maar niet theophylline, stimuleren de sekretie van enzymen. Cyclisch AMP heeft geen effekt op beide parameters. Een bijkomende vinding is, dat bij verschillende kaliumconcentraties in de badvloeistof de concentraties van kalium zowel als natrium in het sekreet vrijwel gelijk zijn aan die in de badvloeistof.

Hoofdstukken III, IV en V beschrijven de karakterisering van de enzymen in de rattepancreas, welke betrokken zijn bij de opbouw en afbraak van cyclisch AMP, respektievelijk het adenylaat cyclase en het cyclisch AMP phosphodiesterase, en de regulatie van de aktiviteiten van deze enzymen. Het adenylaat cyclase is een membraan-gebonden enzym, dat intracellulair ATP omzet in cyclisch AMP. In de rattepancreas blijkt dit enzym, behalve door fluoride, gestimuleerd te worden door lage concentraties van het hormoon secretine. De concentratie, nodig voor half maximale stimulatie, is 1.5×10^{-8} M. Dit is een ondersteuning van de theorie, dat cyclisch AMP intermediair is in het proces van de vloeistof- en elektrolytsekretie. Pancreozymine stimuleert eveneens het adenylaat cyclase, maar op molaire basis is daarvan 100 maal zoveel nodig als van secretine. Acetylcholine heeft geen invloed op

de aktiviteit van het enzym.

Voor de bepaling van het cyclisch AMP phosphodiesterase, welk cyclisch AMP omzet in 5'-AMP, wordt een gemodificeerde methode uitgewerkt, daar de tot nu toe gebruikelijke methodes geen rekening houden met de verdere enzymatische omzetting van het reaktieprodukt, welke omzetting eveneens bestudeerd wordt. Een tot nu toe onbekend afbraakprodukt van cyclisch AMP, nl. cyclisch IMP, wordt aangetoond. Het blijkt, dat er twee phosphodiesterase aktiviteiten kunnen gekarakteriseerd worden in de rattepancreas, een met een hoge, de ander met een lage affiniteit voor het substraat. Beide aktiviteiten worden in verschillende mate geremd door theophylline en papaverine; pancreozymine, secretine en acetylcholine beinvloeden de aktiviteit niet. Aan het enzym met de hoge substraataffiniteit wordt de meeste fysiologische significantie toegeschreven.

In hoofdstuk VI wordt dat deel van de hypothese van Ridderstap en Bonting getoetst, dat stelt, dat cyclisch AMP de enzymsekretie zou stimuleren door het stimuleren van phospholipase A. Cyclisch AMP blijkt evenwel de lipolytische enzymen in de varkenspancreas niet te stimuleren. Wel veroorzaakt het nucleotide een verhoging van de release van enzymen uit geisoleerde zymogeengranula uit de varkenspancreas. Dit effect speelt zich echter af op een relatief lange tijdbasis (meer dan 30 minuten), zodat aan de fysiologische significantie van dit fenomeen getwijfeld kan worden. Een snel verhoogde release wordt geinduceerd door niet gezuiverde commerciele preparaten van secretine en pancreozymine, hetgeen waarschijnlijk toegeschreven kan worden aan verontreinigingen van deze preparaten.

Daar lysofosfolipiden toch een rol zouden kunnen spelen in het beschreven versmeltingsproces, wordt in hoofdstuk VII

de fosfolipidensamenstelling van verschillende subcellulaire fracties uit de varkenspancreas bestudeerd. Een hoog gehalte aan lyso-PC en lyso-PE wordt gevonden, voornamelijk in de membranen van zymogeengranula. Uit experimenten met radioaktief gemerkte fosfolipiden wordt evenwel gekonkludeerd, dat dit verschijnsel op een artefakt berust, veroorzaakt door een snelle post-mortem afbraak van fosfolipiden door lipolytische enzymen.

In verband met literatuurgegevens, dat ook calcium een essentiele rol zou spelen in de enzymsekretie door de pancreas, wordt in hoofdstuk VI tenslotte de sekretie van calcium en magnesium door de geisoleerde konijnepancreas onderzocht. De hoeveelheid gesekreteerde ionen lijkt, althans onder de bestudeerde omstandigheden, te bestaan uit twee komponenten, een basale komponent en een komponent, geassocieerd met de enzymkomponent van het sekreet. Deze laatste komponent wordt bij stimulatie van de enzymsekretie parallel met de eiwitsekretie verhoogd.

In de algemene diskussie tenslotte wordt nagegaan, in hoeverre de hiervoor beschreven hypothese van Ridderstap en bonting is bewezen of ontzenuwd. Het tweede deel van deze hypothese kan als onjuist beschouwd worden. Wat betreft het eerste deel lijkt nader onderzoek noodzakelijk, omdat de beschikbare gegevens elkaar tegenspreken. Naast cyclisch AMP zouden andere faktoren de enzymsekretie mede kunnen reguleren. Met name de rol van calcium in deze regulatie dient nader onderzocht te worden. In dit verband wordt nog aandacht besteed aan de hypothese van Rasmussen et al. (1972), die zowel calcium als cyclisch AMP als "second messengers" postuleert. Ook deze hypothese evenwel dient nog getoetst.

Ahkong, Q.F., Cramp, F.C., Fisher, D., Howell, J.I. and Lucy, J.A., (1972): Studies on chemically induced cell fusion, J. Cell Sci., 10, 769-787

Argent, B.E., Case, R.M. and Scratcherd, T., (1971). Stimulation of amylase secretion from the perfused cat pancreas by potassium and other alkali metal ions, J. Physiol., 216, 611-624

Argent, B.E., Case, R.M. and Scratcherd, T., (1973): Amylase secretion by the perfused cat pancreas in relation to the secretion of calcium and other electrolytes and as influenced by the external ionic environment, J. Physiol., 230, 575-593

Ashcroft, S.J.H., Randle, P.J. and Taljedal, I.-B., (1972): Cyclic nucleotide phosphodiesterase activity in normal mouse pancreatic islets, FEBS Lett., 20, 263-266

Ball, E.G. and Johnston, C.G., (1930): Variations in inorganic constituents of the pancreatic juice during constant drainage of the pancreatic ducts, J. Biol. Chem., 86, 643-653

Bär, H.P. and Hechter, O., (1969): Adenyl Cyclase Assay in Fat Cell Ghosts, Anal. Biochem., 29, 476-489

Bauduin, H., Rochus, L., Vincent, D. and Dumont, J.E., (1971): Role of cyclic 3',5'-AMP in the action of physiological secretagogues on rat pancreas in vitro, Biochim. Biophys. Acta, 252, 171-183

Bauduin, H. and Cantraine, F., (1972): "Phospholipid effect" and secretion in the rat pancreas, Biochim. Biophys. Acta, 270, 248-253

Bdolah, A., Ben-Zvi, R. and Schramm, R., (1964): The Mechanism of Enzyme Secretion by the Cell. II. Secretion of Amylase and Other Proteins of Rat Parotid Gland, Arch. Biochem. Biophys., 104, 58-66

Bdolah, A. and Schramm, M., (1965): The function of 3',5' cyclic AMP in enzyme secretion, Biochem. Biophys. Res. Comm., 18, 452-455

Beavo, J.A., Hardman, J.G. and Sutherland, E.W., (1970): Hydrolysis of Cyclic Guanosine and Adenosine 3',5'-Monophosphates by Rat and Bovine Tissues, J. Biol. Chem., 245, 5649-5655

Beavo, J.A., Hardman, J.G. and Sutherland, E.W., (1971): Stimulation of Adenosine 3',5'-Monophosphate Hydrolysis by Guanosine 3',5'-Monophosphate, J. Biol. Chem., 246, 3841-3846

Benz, L., Eckstein, B., Matthews, E.K. and Williams, J.A., (1972): Control of pancreatic amylase release in vitro: effects of ions, cyclic AMP and colchicine, Brit. J. Pharmacol., 46, 66-77

Bernfeld, P., (1955), In: Methods in Enzymology, ed. by S.P. Colowick and N.O. Kaplan, New York, Acad. Press, p. 149

Birnbaumer, L., Pohl, S.L. and Rodbell, M., (1971): The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver. II. Comparison between glucagon and fluoride-stimulated activities, J. Biol. Chem., 246, 1857-1860

Blair., E.L., Brown, J.C., Harper, A.A. and Scratcherd, T., (1966): A gastric phase of pancreatic secretion, J. Physiol., 184, 812-824

Blaschko, H., Firemark, H., Smith, A.D. and Winkler, H., (1967): Lipids of the Adrenal Medulla. Lysolecithin, a characteristic constituent of chromaffin granules, Biochem. J., 104, 545-549

Broekhuyze, R.M., (1968): Phospholipids in tissues of the eye I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids, Biochim. Biophys. Acta, 152, 307-315

Brooker, G., Thomas, L.J. and Appleman, M.M., (1968): The Assay of Adenosine 3',5'-Cyclic Monophosphate and Guanosine 3',5'-Cyclic Monophosphate in Biological Materials by Enzymatic Radioisotopic Displacement, Biochemistry, 12, 4177-4181

Brown, J.C., Harper, A.A. and Scratcherd, T., (1967): Potentiation of secretin stimulation of the pancreas, J. Physiol., 190, 519-530

Burwen, S.J. and Rothman, S.S., (1972): Zymogen granules· osmotic properties, interactions with ions, and some structural implications, Amer. J. Physiol., 222, 1177-1181

Butcher, R.W. and Sutherland, E.W., (1962): Adenosine 3',5'-Phosphate in Biological Materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine, J. Biol. Chem., 237, 1244-1250

Carvalho, A.P., (1968): Calcium-Binding Properties of Sarcoplasmatic Reticulum As Influenced by ATP, Caffeine, Quinine, and Local Anesthetics, J. Gen. Physiol., 52, 622-642

Case, R.M., (1973a): Cellular mechanisms controlling pancreatic exocrine secretion, Acta Hepato-Gastroenterologica, 20, 435-443

Case, R.M., (1973b): Calcium and Gastrointestinal Secretion, Digestion, 8, 269-288

Case, R.M. and Clausen, T., (1973): The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas, J. Physiol., 235, 75-102

Case, R.M., Harper, A.A. and Scratcherd, T., (1968): Water and electrolyte secretion by the perfused pancreas of the cat, J. Physiol., 196, 133-149

Case, R.M., Laundy, T.J. and Scratcherd, T., (1969): Adenosine 3',5' monophosphate (cyclic AMP) as the intracellular mediator of the action of secretin on the exocrine pancreas, J. Physiol., 204, 45P-47P

Case, R.M., Johnson, M., Scratcherd, T. and Sherratt, H.S.A., (1972): Cyclic adenosine 3',5'-monophosphate concentration in the pancrea. following stimulation by secretin, cholecystokinin-pancreozymin and acetylcholine, J. Physiol., 223, 669-684

Case, R.M. and Scratcherd, T., (1972): The actions of dibutyryl cyclic adenosine 3',5'-monophosphate and methyl xanthines on pancreatic exocrine secretion, J. Physiol., 223, 649-667

Cheung, W.Y., (1971). Cyclic 3',5'-nucleotide phosphodiesterase. Effect of divalent cations, Biochim. Biophys. Acta, 242, 395-409

Chiappe de Cingolani, G.E., Van Den Bosch, H. and Van Deenen, L.L.M., (1972): Phospholipase A and lysophospholipase activities in isolated fat cells: effects of cyclic 3',5'-AMP

Coddington, A., (1965): Some substrates and inhibitors of adenosine deaminase, Biochim. Biophys. Acta, 99, 442-451

Dean, P.M. and Matthews, E.K., (1972)· Pancreatic acinar cells: measurements of membrane potential and miniature depolarization potentials, J. Physiol., 225, 1-13

De Haas, G.H., Postema, N.M., Nieuwenhuizen, W. and Van Deenen, L.L.M., (1968a): Purification and properties of phospholipase A from porcine pancreas, Biochim. Biophys. Acta, 159, 103-117

De Haas, G.H., Postema, N.M., Nieuwenhuizen, W. and Van Deenen, L.L.M., (1968b): Purification and properties of an anionic zymogen of phospholipase A from porcine pancreas, Biochim. Biophys. Acta, 159, 118-129

Desnuelle, P., Sarda, L. and Ailhaud, G., (1960): Inhibition de la lipase pancréatique par le diethyl-p-nitrophenyl phosphate en emulsion, Biochim. Biophys. Acta, 37, 570-571
Diamond, J.M., (1962): The mechanism of water transport by the gall bladder, J. Physiol., 161, 503-527
Dole, V.P., (1956): Fatty acid analysis in biological materials, J. Clin. Invest., 35, 150-155
Dousa, T. and Rychlík, I., (1970): The metabolism of adenosine 3',5'-cyclic phosphate. II. Some properties of adenosine-3',5'-cyclic phosphate phosphodiesterase from the rat kidney, Biochim. Biophys. Acta, 204, 10-17
Dreiling, D.A. and Janowitz, H.A., (1956): The secretion of electrolytes by the human pancreas, Gastroenterology, 30, 382-390
Dyck, W.P., Rudick, J., Hoexter, B. and Janowitz, H.D., (1969): Influence of glucagon on pancreatic exocrine secretion, Gastroenterology, 56, 531-537
Ekholm, R. and Edlund, Y., (1959): Ultrastructure of the human exocrine pancreas, J. Ultrastructure Res., 2, 453-481
Feinstein, H. and Schramm, M., (1970): Energy Production in Rat Parotid Gland. Relation to Enzyme secretion and Effects of Calcium, Eur. J. Biochem., 13, 158-163
Fleischer, N., Donald, R.A. and Butcher, R.W., (1969): Involvement of adenosine 3',5' monophosphate in release of ACTH, Amer. J. Physiol., 217, 1287-1291
Folch, J., Lees, M. and Sloane-Stanley, G.H., (1957): A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem., 226, 497-509
Foreman, J.C., Mongar, J.L. and Gomperts, B.D., (1973): Calcium Ionophores and Movement of Calcium Ions following the Physiological stimulus to a Secretory Process, Nature, 245, 249-251
Franks, D.J. and MacManus, J.P., (1971): Cyclic GMP stimulation and inhibition of cyclic AMP phosphodiesterase from thymic lymphocytes, Biochem. Biophys. Res. Comm., 42, 841-849
George, W.J., Polson, J.B., O'Toole, A.G. and Goldberg, N.D., (1970): Elevation of Guanosine 3',5'-Cyclic Phosphate in Rat Heart after Perfusion with Acetylcholine, Proc. Natl. Acad. Sci. U.S., 66, 398-403
Goebell, H., Steffen, Ch. and Bode, Ch., (1972): Stimulatory effect of pancreozymin-cholecystokinin on calcium secretion in pancreatic juice of dogs, Gut, 13, 477-482
Grossman, M.I., (1967): In: Gastric Secretion Mechanisms and Control, ed. by T.K. Shitka, J.A.C. Gilbert and R.C. Harrison, Pergamom Press, Oxford, p. 186
Grossman, M.I., (1970): Effects of gastrin, cholecystokinin and secretin on gastric and pancreatic secretion: a theory of interaction of hormones; In: Origin, Chemistry and Physiology of the Gastrointestinal hormones, ed. by W. Creutzfeldt, Schattauer Verlag, Stuttgart, p. 129
Grossman, M.I., (1971): Control of pancreatic secretion; In: The Exocrine Pancreas: Proceedings of a Symposium held at Queen's University, Kingston, Ontario, June 1969, ed. by I.T. Beck and D.G. Sinclair, Churchill, London, ch. 5, p. 59

Grossman, M.I. and Ivy, A.C., (1946): Effect of alloxan upon external secretion of pancreas, Proc. Soc. Exptl. Biol. Med., 63, 62-63
Gottfried, E.L. and Rapport, M.M., (1963): The biochemistry of plasmalogens: II. Hemolytic activity of some plasmalogen derivatives, J. Lipid Res., 4, 57-62
Gulyassy, P.F. and Oken, R.L., (1971): Assay of Cyclic 3',5'-Nucleotide Phosphodiesterase in Tissue Homogenates, Proc. Soc. Exptl. Biol. Med., 137, 361-365
Harper, A.A., (1967): Hormonal control of pancreatic secretion; In: Handbook of Physiology, Section 6: Alimentary Canal, Volume II: Secretion, ed. by W. Heidel, American Physiological Society, Washington, D.C., p. 969-995
Harper, A.A., (1972): The control of pancreatic secretion, Gut, 317, 308-317
Harper, A.A., Kidd, C. and Scratcherd, T., (1959): Vago-vagal reflex effects on gastric and pancreatic secretion and gastro-intestinal motility, J. Physiol., 148, 417-437
Heatley, N.G., (1968). The assay of secretin in the rat, J. Endocrinol., 42, 535-547
Hegre, O.D., McEvoy, R.C., Bachelder, V. and Lazarow, A., (1972): In vitro organ culture of fetal rat pancreas. Quantitative analysis by linear scanning of islet and other tissue components, J. Tissue Cult. Ass., 7, 366-377
Heisler, S., Fast, P. and Tenenhouse, A., (1972): Role of Ca^{2+} and cyclic AMP in protein secretion from rat exocrine pancreas, Biochim. Biophys. Acta, 279, 561-572
Hickson, J.C.D., (1970): The secretion of pancreatic juice in response to stimulation of the vagus nerves in the pig, J. Physiol., 216, 275-297
Hokin, L.E., (1955): Isolation of zymogen granules of dog pancreas and a study of their properties, Biochim. Biophys. Acta, 18, 379-388
Hokin, L.E., (1966): Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices, Biochim. Biophys. Acta, 115, 219-221
Hokin, L.E. and Hokin, M.R., (1955). Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices, Biochim. Biophys. Acta, 18, 102-110
Hokin, L.E. and Sherwin, A.L., (1957): Protein secretion and phosphate turnover in salivary glands in vitro, J. Physiol., 135, 18-29
Hokin, M.R., Benfey, B.G. and Hokin, L.E., (1958): Phospholipids and Adrenaline Secretion in Guinea-Pig Adrenal Medulla, J. Biol. Chem., 233, 814-817
Hokin, M.R. and Hokin, L.E., (1953): Enzyme secretion and the incorporation of P^{32} into phospholipids of pancreas slices, J. Biol. Chem., 203, 967-977
Howell, J.I. and Lucy, J.A., (1969): Cell fusion induced by lysolecithin, FEBS-Lett., 4, 147-150
Huang, Y.-C. and Kemp, R.G., (1971): Properties of a Phosphodiesterase with High Affinity for Adenosine 3',5'-Cyclic Phosphate, Biochemistry, 10, 2278-2283
Hubel, K.A., (1970) Response of rabbit pancreas in vitro to adrenergic agonists and antagonists, Amer. J. Physiol., 219, 1590-1594

Ishida, H., Miki, N. and Yoshida, (1971a): Role of Ca^{2+} in the secretion of amylase from the parotid gland, Japan. J. Pharmacol., 21, 227-238

Ishida, H., Miki, N., Hata, I. and Yoshida, H., (1971b): Conformational changes in zymogen granules from the parotid glands induced by ATP and a low concentration of calcium, Japan. J. Pharmacol., 21, 239-251

Ichikawa, A., (1965): Fine structural changes in response to hormonal stimulation of the perfused canine pancreas, J. Cell Biol., 24, 369-385

Jamieson, J.A. and Palade, G.E., (1967a): Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the Peripheral Elements of the Golgi Complex, J. Cell Biol., 34, 577-596

Jamieson, J.D. and Palade, G.E., (1967b): Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to Condensing Vacuoles and Zymogen Granules, J. Cell Biol., 34, 597-615

Jamieson, J.D. and Palade, G.E., (1968a): Intracellular transport of secretory proteins in the pancreatic exocrine cell. III. Dissociation of Intracellular Transport from Protein Synthesis, J. Cell Biol., 39, 580-588

Jamieson, J.D. and Palade, G.E., (1968b): Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic Requirements, J. Cell Biol., 39, 589-603

Jamieson, J.D. and Palade, G.E., (1971a): Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: metabolic studies, J. Cell Biol., 48, 503-522

Jamieson, J.D. and Palade, G.E., (1971b): Synthesis, intracellular transport and discharge of secretory proteins in stimulated pancreatic cells, J. Cell Biol., 50, 135-158

Janowitz, H.D., (1967): Pancreatic secretion of fluid and electrolytes; In: Handbook of Physiology, Section 6: Alimentary Canal, Volume II: Secretion, ed. by W. Heidel, American Physiological Society, Washington, D.C., p. 925-933

Jard, S. and Bernard, S., (1970) Presence of two 3'-5'-cyclic AMP phosphodiesterases in rat kidney and frog bladder epithelial cells extracts, Biochem. Biophys. Res. Comm., 41, 781-788

Johnson, M., Sherratt, H.S.A., Case, R.M. and Scratcherd, T., (1971): The Effects of Secretin, Pancreozymin and Acetylcholine on the Concentration of Adenosine 3' 5'-Cyclic Monophosphate in Cat Pancreas, Biochem. J., 120, 8P-9P

Kalser, M.H. and Grossman, M.I., (1954): Pancreatic secretion in dogs with ethionine-induced pancreatitis, Gastroenterology, 26, 189-197

Kakiuchi, S. and Yamazaki, R., (1970a): Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. Studies on Cyclic 3',5'-Nucleotide Phosphodiesterase (III), Biochem. Biophys. Res. Comm., 41, 1104-1110

Kakiuchi, S. and Yamazaki, R., (1970b)· Stimulation of the Activity of Cyclic 3',5'-Nucleotide Phosphodiesterase by Calcium Ion, Proc. Japan. Acad., 46, 387-392

Kakiuchi, S., Yamazaki, R. and Teshima, Y., (1971): Cyclic 3',5'-Nucleotide Phosphodiesterase, IV. Two Enzymes with Different Properties from Brain, Biochem. Biophys. Res. Comm., 42, 968-974

Kanno, T., (1972) Calcium dependent amylase release and electrophysiological measurements in cells of the pancreas, J. Physiol., 226, 353-371
Kanno, T., Cochrane, D.E. and Douglas, W.W., (1973) Exocytosis (Secretory Granule Extrusion) Induced by Injection of Calcium into Mast Cells, Canad. J. Physiol. Pharmacol., 51, 1001-1004
Kaye, G.I., Wheeler, H.O., Whitlock, R.T. and Lane, N., (1966): Fluid transport in the rabbit gall bladder. A combined physiological and electronmicroscopic study, J. Cell Biol., 30, 237-268
Kern, H.F. and Ferner, H., (1971) Die Feinstruktur des exokrinen Pankreasgewebe vom Menschen, Z. Zellforsch., 113, 322-343
King, E.T., (1967) In Methods in Enzymology, ed. by S.P. Colowick and N.O. Kaplan, New York, Acad. Press, Vol. X, p. 322-331
Klotz, U., Berndt, S. and Stock, K., (1972): Characterization of multiple cyclic nucleotide phosphodiesterase activities of rat adipose tissue, Life Sci., 11 II, 7-17
Knodell, R.G., Toskes, P.P., Reber, H.A. and Brooks, F.P., (1970): Significance of Cyclic AMP in the Regulation of Exocrine Pancreas Secretion, Experientia, 26, 515-517
Kramer, M.F. and Poort, C., (1968): Protein Synthesis in the Pancreas of the Rat after Stimulation of Secretion, Z. Zellforsch., 86, 475-486
Kramer, M.F. and Poort, C., (1972) Unstimulated secretion of protein from rat exocrine pancreas cells, J. Cell Biol., 52, 147-158
Kulka, R.G. and Sternlicht, E., (1968)· Enzyme secretion in mouse pancreas mediated by adenosine-3',5'-cyclic phosphate and inhibited by adenosine-5'-phosphate, Proc. Natl. Acad. Sci. U.S., 61, 1123-1128
Kuo, J.F. and Greengard, P., (1969). Cyclic nucleotide-dependent protein kinases. IV. Widespread occurrence of adenosine 3',5'-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom, Proc. Natl. Acad. Sci. U.S., 64, 1349-1353
Kuo, J.F., Lee, T.P., Reyes, P.L., Walton, K.G., Donnelly, T.E. and Greengard, P., (1972): Cyclic Nucleotide-dependent Protein Kinases. X. An assay method for the measurement of guanosine 3',5'-monophosphate in various biological materials and a study of agents regulating its levels in heart and brain, J. Biol. Chem., 247, 16-22
Lehninger, A.L., (1970)· Mitochondria and calcium ion transport, Biochem. J., 119, 129-138
Lemay, A. and Labrie, F., (1972): Calcium dependent stimulation of prolactin release in rat anterior pituitary in vitro by N^6-monobutyryl adenosine 3',5' monophosphate, FEBS-Lett.; 20, 7-10
Lenninger, S. and Ohlin, P., (1971)· The flow of juice from the pancreatic gland of the cat in response to vagal stimulation, J. Physiol., 216, 303-318
Levine, R.A., (1970): The role of cyclic AMP in hepatic and gastrointestinal function, Gastroenterology, 59, 280-300
Lishajko, F., (1971)· Studies on catecholamine release and uptake in adreno-medullary storage granules, Acta Physiol. Scand., Supplementum 362
Lockhart Ewart, R.B. and Taylor, K.W., (1971)· The Regulation of Growth Hormone Secretion from the Isolated Rat Anterior Pituitary in vitro, Biochem. J., 124, 815-826

Loten, E.G. and Sneyd, J.G.T., (1970): An Effect of Insulin on Adipose Tissue Adenosine 3':5'-Cyclic Monophosphate Phosphodiesterase, Biochem. J., 120, 187-193
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., (1951): Protein measurement with the Folin Phenol reagent, J. Biol. Chem., 193, 265-275
Lucy, J.A., (1970): The Fusion of Biological Membranes, Nature, 227, 814-817
Malaisse, W.J., Malaisse-Lagae, F., Walker, M.O. and Lucy, P.E., (1971): The stimulus-secretion coupling of glucose-induced insulin release. V. The participation of a microtubular-microfilamentous system, Diabetes, 20, 257-265
Marchis-Mouren, G., Sarda, L. and Desnuelle, P., (1959): Purification of Hog Pancreatic Lipase, Arch. Biochem. Biophys., 83, 309-319
Meldolesi, J. and Cova, D., (1971): In vitro stimulation of enzyme secretion and the synthesis of microsomal membranes in the pancreas of the guinea-pig, J. Cell Biol., 51, 396-404
Meldolesi, J., Jamieson, J.D. and Palade, G.E., (1971a): Composition of cellular membranes in the pancreas of the guinea pig. I. Isolation of Membrane Fractions, J. Cell Biol., 49, 109-129
Meldolesi, J., Jamieson, J.D. and Palade, G.E., (1971b): Composition of cellular membranes in the pancreas of the guinea pig. II. Lipids, J. Cell Biol., 49, 130-149
Miki, N. and Yoshida, H., (1972): Purification and properties of cyclic AMP phosphodiesterase from rat brain, Biochim. Biophys. Acta, 268, 166-174
Montgomery, M.L., Sheline, G.E. and Chaikoff, I.L., (1941): Elimination of sodium in pancreatic juice as measured by radioactive sodium, Amer. J. Physiol., 131, 578-583
Morisset, J. and Webster, P.D., (1970): Effects of atropine on pigeon pancreas, Amer. J. Physiol., 219, 1286-1291
Morisset, J. and Webster, P.D., (1971): In vitro and in vivo effects of pancreozymin, urecholine, and cyclic AMP on rat pancreas, Amer. J. Physiol., 230, 202-208
Morley, J.S., Tracy, H.J. and Grgory, R.A., (1965): Structure-function Relationships in the active C-terminal tetrapeptide sequence of gastrin, Nature, 207, 1356-1359
Morrison, W.R. and Smith, L.M., (1964): Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol, J. Lipid Res., 5, 600-608
Murad, F., Chi, Y.H., Rall, T.W. and Sutherland, E.W., (1962): Adenyl Cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3',5'-phosphate by preparations from cardiac muscle and liver, J. Biol. Chem., 237, 1233-1239
Mutt, V., (1964): Behavior of secretin, cholecystokinin and pancreozymin to oxidation with H_2O_2, Acta Chem. Scand., 18, 2185-2186
Mutt, V. and Jorpes, E., (1967): Contemporary developments in the biochemistry of the gastrointestinal hormones, Recent Prog. Hormone Res., 23, 483-503
Nair, K.G., (1966): Purification and Properties of 3',5'-Cyclic Nucleotide Phosphodiesterase from Dog Heart, Biochemistry, 5, 150-157

Neve, P. and Dumont, J.E., (1970): Effects in vitro of thyrotropin, cyclic 3',5' AMP, dibutyryl cyclic 3',5' AMP, and prostaglandin E_1 on the ultrastructure of dog thyroid slices, Exptl.Cell Res., 63, 285-292

Palade, G.E., (1959): In: Subcellular Particles, ed. by T. Hayashi, The Ronal Press Company, New York, p. 64

Petersen, O.H. and Matthews, E.K., (1972): The effect of pancreozymin and acetylcholine on the membrane potential of the pancreatic acinar cell, Experientia, 28, 1037-1038

Pöch, G., (1971): Assay of Phosphodiesterase with Radioactively Labeled Cyclic 3',5'-AMP as Substrate, Naunyn-Schmiedebergs Arch. Exp. Path. Pharmakol., 268, 272-299

Pohl, S.L., Birnbaumer, L. and Rodbell, M., (1971): The Glucagon-Sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver, J. Biol. Chem., 246, 1849-1856

Preshaw, R.M., (1967). Integration of nervous and hormonal mechanisms for external pancreatic secretion; In: Handbook of Physiology, Section 6: Alimentary Canal, Volume II Secretion, ed. by W. Heidel, American Physiological Society, Washington, D.C., p. 997-1005

Preshaw, R.M., Cooke, A.R. and Grossman, M.I., (1966): Sham feeding and pancreatic secretion in the dog, Gastroenterology, 50, 171-178

Putzke, H.-P. and Ernst, B., (1971): Chemisch-analytische und elektronenmikroskopische Untersuchungen zur 5'-Nukleotidase-Aktivität im exokrinen Parenchym und Sekret des Rattenpankreas, Acta biol. med. germ., 26, 665-670

Rasmussen, H., (1970): Cell communication, calcium ion and cyclic adenosine monophosphate, Science, 170, 404-412

Rasmussen, H., Goodman, D.B.P. and Tenenhouse, A., (1972): The role of cyclic AMP and Calcium in cell activation, CRC Critical Reviews in Biochemistry, 1972, 95-148

Rasmussen, H. and Tenenhouse, A., (1968): Cyclic adenosine monophosphate, Ca^{2+} and membranes, Proc. Natl. Acad. Sci. U.S., 59, 1364-1369

Ratner, A., (1970): Stimulation of luteinizing hormone release in vitro by dibutyryl cyclic AMP and theophylline, Life Sci., 9 I, 1221-1226

Reggio, H., Cailla Deckmyn, H. and Marchis-Mouren, G., (1971): Effect of pancreozymin on rat pancreatic enzyme biosynthesis, J. Cell Biol., 50, 333-343

Reman, F.C., Demel, R.A., De Gier, J., Van Deenen, L.L.M., Eibl, H. and Westphal, O., (1969): Studies on the lysis of red cells and bimolecular lipid leaflets by synthetic lysolecithins, lecithins and structural analogues, Chem. Phys. Lipids, 3, 221-233

Ridderstap, A.S., (1969): Mechanisms involved in exocrine pancreatic secretion, Thesis, University of Nijmegen, Nijmegen, p. 95-102

Ridderstap, A.S. and Bonting, S.L., (1969a): Na^+-K^+-activated ATPase and exocrine pancreatic secretion in vitro, Amer. J. Physiol., 217, 1721-1727

Ridderstap, A.S. and Bonting, S.L., (1969b): Enzyme Secretion by the Isolated Rabbit Pancreas. Absence of a Relationship with the Na-K activated ATPase, Pflügers Arch., Eur. J. Physiol., 313, 53-61

Ridderstap, A.S. and Bonting, S.L., (1969c): Cyclic AMP and Enzyme Secretion by the Isolated Rabbit Pancreas, Pflügers Arch., Eur. J. Physiol., 313, 62-70

Robertson, J.D., (1961): In. Electron Microscopy in Anatomy, ed. by J.D. Boyd, F.R. Johnson and J.D. Lever, Baltimore, Williams and Williams, p. 74
Robberecht, P. and Christophe, J., (1971): Secretion of hydrolases by perfused fragments of rat pancreas: effect of calcium, Amer. J. Physiol., 220, 911-917
Rodbell, M., Birnbaumer, L. and Pohl, S.L., (1970) Adenyl Cyclase in Fat Cells. III. Stimulation by secretin and the effects of trypsin on the receptors for lipolytic hormones, J. Biol. Chem., 245, 718-722
Rothman, S.S., (1964): Exocrine Secretion from the isolated Rabbit Pancreas, Nature, 204, 84-85
Rothman, S.S., (1971)· The behavior of isolated zymogen granules: pH-dependent release and reassociation of protein, Biochim. biophys. Acta, 241, 567-577
Rothman, S.S. and Brooks, F.P., (1965): Electrolyte secretion from rabbit pancreas in vitro, Amer. J. Physiol., 208, 1171-1176
Rubin, R.P., (1970): The role of calcium in the release of neurotransmitter substances and hormones, Pharmacol. Rev., 22, 389-428
Rutten, W.J., De Pont, J.J.H.H.M. and Bonting, S.L., (1972): Adenylate cyclase in the rat pancreas. Properties and stimulation by hormones, Biochim. Biophys. Acta, 274, 201-213
Rutten, W.J., Schoot, B.M. and De Pont, J.J.H.H.M., (1973a): Adenosine 3',5'-monophosphate phosphodiesterase assay in tissue homogenates, Biochim. Biophys. Acta, 315, 378-383
Rutten, W.J., Schoot, B.M., De Pont, J.J.H.H.M. and Bonting, S.L.,(1973b), Adenosine 3',5'-monophosphate phosphodiesterase in rat pancreas, Biochim. Biophys. Acta, 315, 384-393
Santhanam, K. and Wagle, S.R., (1971): Studies on in vitro activation of high molecular weight pancreatic lipase, Biochem. Biophys. Res. Comm., 43, 1369-1375
Sarles, H., Dani, R., Prezelin, G., Souville, C. and Figarella, C., (1968): Cephalic phase of pancreatic secretion in man, Gut, 9, 214-221
Schramm, M., (1967). Secretion of enzymes and other macromolecules, Annu. Rev. Biochem., 36, 307-320
Schramm, M. and Naim, E., (1970) Adenyl Cyclase of Rat Parotid Gland. Activation by fluoride and norepinephrine, J. Biol. Chem., 245, 3225-3231
Schultz, G., Senft, G., Losert, W. and Sitt, R., (1966): Biochemische Grundlagen der Diazoxid-Hyperglykämie, Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol., 18, 372-387
Schwabe, U., Berndt, S. and Ebert, R., (1972): Activation and Inhibition of Lipolysis in Isolated Fat Cells by Various Inhibitors of Cyclic AMP Phosphodiesterase, Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol., 273, 62-74
Shapiro, B., (1953)· Purification and Properties of a Lysolecithinase from Pancreas, Biochem. J., 53, 663-666
Siekevitz, P. and Palade, G.E., (1958): A cytochemical study on the pancreas of the guinea pig. III. In vivo incorporation of leucine 1-C14 into the proteins of cell fractions, J. Biophys. Biochem. Cytol., 4, 557-566
Simpson, L.L., (1968): The role of calcium in neurohumoral and neurohormonal extrusion processes, J. Pharm. Pharmacol., 20, 889-910

Sjöstrand, F.S. and Elfvin, L.G., (1962) The layered, asymmetric structure of the plasma membrane in the exocrine pancreas cells of the cat, J. Ultrastructure Res., 7, 504-510
Skidmore, W.D. and Entenman, C., (1962)· Two-dimensional thin-layer chromatography of rat liver phosphatides, J. Lipid Res., 3, 471-475
Skipski, V.P., Peterson, R.F. and Barclay, M., (1964)· Quantitative Analysis of Phospholipids by Thin-Layer Chromatography, Biochem. J., 90, 374-377
Slotboom, A.J., De Haas, G.H., Bonsen, P.P.M., Burbach-Westerhuis, G.D. and Van Deenen, L.L.M., (1970)· Hydrolysis of phosphoglycerides by purified lipase preparations. I. Substrate-, positional and stereo-specificity, Chem. Phys. Lipids, 4, 15-29
Song, S.-Y. and Cheung, W.Y., (1971): Cyclic 3',5'-nucleotide phospho-diesterase. Properties of the enzyme of human blood platelets, Biochim. Biophys. Acta, 242, 593-605
Spears, G., Sneyd, J.G.T. and Loten, E.G., (1971): A Method for Deriving Kinetic Constants for Two Enzymes Acting on the Same Substrate, Biochem. J., 125, 1149-1151
Sussmann, K.E. and Vaughan, G.D., (1967): Insulin release after ACTH, glucagon and adenosine 3',5' phosphate (cyclic AMP) in the perfused isolated rat pancreas, Diabetes, 16, 449-454
Therriault, D.G. and Winters, V.G., (1970): Enzymic hydrolysis of adenosine 3',5'-monophosphate by rat and beef heart supernatant, Life Sci., 9 II, 1053-1060
Thomas, J.E., (1967): Neural regulation of pancreatic secretion; In: Handbook of Physiology, Section 6: Alimentary Canal, Volume II: Secretion, ed. by W. Heidel, American Physiological Society, Washington, D.C., p. 955-968
Thompson, W.J. and Appleman, M.M., (1971a): Multiple Cyclic Phospho-diesterase Activities from Rat Brain, Biochemistry, 10, 311-316
Thompson, W.J. and Appleman, M.M., (1971b): Characterization of Cyclic Nucleotide Phosphodiesterases of Rat Tissues, J. Biol. Chem., 246, 3145-3150
Tonoue, T., Tong, W. and Stolc, V., (1970): TSH and dibutyryl cyclic AMP Stimulation of Hormone Release from Rat Thyroid Glands in vitro, Endocrinology, 86, 271-277
Triner, L., Vulliemoz, Y., Schwartz, I. and Nahas, G.G., (1970): Cyclic phosphodiesterase activity and the action of papaverine, Biochem. Biophys. Res. Comm., 40, 64-69
Turtle, J.R. and Kipnis, D.M., (1967): An adrenergic receptor mechanism for the control of cyclic 3',5' adenosine monophosphate in tissues, Biochem. Biophys. Res. Comm., 169, 80-86
Van Den Bosch, H., Aarsman, A.J., Slotboom, A.J. and Van Deenen, L.L.M., (1968). On the specificity of rat-liver lysophospholipase, Biochim. Biophys. Acta, 164, 215-225
Van Den Bosch, H., Postema, N.M., De Haas, G.H. and Van Deenen, L.L.M., (1965): On the positional specificity of phospholipase A from pancreas, Biochim. Biophys. Acta, 98, 657-659
Wade, D.R., Chalmers, T.M. and Hales, C.N., (1970): The effects of Mg^{2+},Ca^{2+} , ATP and cyclic 3',5'-AMP on the hormone-sensitive lipase of adipose tissue, Biochim. Biophys. Acta, 218, 496-507
Wang, C.C. and Grossman, M.I., (1951). Physiological determination of the release of secretin and pancreozymin from intestine of dogs with transplanted pancreas, Amer. J. Physiol., 164, 527-545

Webster, P.D., Gunn, L.D. and Tyor, M.P., (1966) Effect of in vivo pancreozymin and methacholine on pancreatic lipid metabolism, Amer. J. Physiol., 211, 781-785
Webster, P.D. and Tyor, M.P., (1966) Effect of intravenous pancreozymin on amino acid incorporation in vitro by pancreatic tissue, Amer. J. Physiol., 211, 157-160
Weinstein, B., (1968) On the Relationship Between Glucagon and Secretin, Experientia, 24, 406-408
Weiss, B., Lehne, R. and Strada, S., (1972) Rapid Microassay of Adenosine 3',5'-Monophosphate Phosphodiesterase Activity, Anal. Biochem., 45, 222-235
White, A., Handler, P. and Smith, E.L., (1968) In Principles of Biochemistry, McGraw-Hill Book Company, New York, p. 779
White, D.A. and Hawthorne, J.N., (1970) Zymogen Secretion and Phospholipid Metabolism in the Pancreas. Phospholipids of the zymogen granule, Biochem. J., 120, 533-538
White, T.T., Lundh, G. and Magee, D.F., (1960): Evidence for the existence of a gastropancreatic reflex, Amer. J. Physiol., 198, 725-728
Williams, J.A. and Wolff, J., (1971): Multiple Actions of Agents affecting Thyroid Secretion, Endocrinology, 88, 206-217
Wills, E.D., (1960): The relation of metals and -SH groups to the activity of pancreatic lipase, Biochim. Biophys. Acta, 40, 570-571
Woods, W.D. and Waitzman, M.B., (1970): Isolation of cyclic 3',5'-adenosine monophosphate on a neutral silicic acid-glass microfiber matrix, J. Chromatogr., 47, 536-542
Wormsley, K.G., Mahoney, M.P. and Ng, M., (1966): Effects of a gastrin-like pentapeptide (I.C.I. 50,123) on stomach and pancreas, Lancet, I, 1966, 993-996
Yang, J. and Dickman, S.R., (1966) Effect of Pancreozymin, Urecholine and Actinomycin D on the Metabolism of Ribonucleic Acid by Canine Pancreas, Biochem. J., 100, 548-555
Zimmerman, M.J., Dreiling, D.A., Rosenberg, I.R. and Janowitz, H.D., (1967): Secretion of calcium by the canine pancreas, Gastroenterology, 52, 865-870
Zor, U., Kaneko, T., Schneider, P.G., McCann, S.M., Lowe, I.P., Bloom, G. and Borland, B., (1969): Stimulation of anterior pituitary adenyl cyclase activity and adenosine 3':5'-cyclic phosphate by hypothalamic extract and prostaglandin E_1, Proc. Natl. Acad. Sci. U.S., 63, 918-925

The following reagents and materials have been used from the indicated sources.

Boehringer, Mannheim, G.F.R.: adenosine-5'-triphosphate disodium salt; adenosine-3':5'-monophosphoric acid (cyclic AMP, 3',5'-AMP); guanosine-3':5'-monophosphate monosodium salt; N^6-2'-O-dibutyryl-adenosine-3':5'-monophosphate monosodium salt; pyruvate kinase from rabbit muscle, crystalline suspension, 10 mg/ml,

Sigma Chemical Co., St. Louis, Mo., U.S.A.: phosphoenolpyruvate trisodium salt; soybean trypsin inhibitor, chromatographically prepared; 5'-nucleotidase from Crotalus adamanteus venom, Grade II; para-choromercuribenzoic acid; phenazine methosulfate; clinical preparations of secretin and pancreozymin.

The Radiochemical Centre, Amersham, England: [α-^{32}P]-adenosine triphosphoric acid, 50 - 300 mCi/mmol; [8-^{3}H]-adenosine 3',5'-cyclic phosphate, ammonium salt, 6.5 Ci/mmol.

Merck A.G., Darmstadt, G.F.R.: silicagel; silicagel HR; 2,6-dichlorophenolindophenol sodium salt.

Bio-Rad Laboratories, Richmond, Calif., U.S.A.: Dowex AG 1-X2, 200 - 400 mesh, chloride form.

Boots pure Drug Co. Ltd., Nothingham, England: clinical preparations of secretin and pancreozymin.

ICI, Macclesfield, England: pentagastrin (Peptavlon^R)

Eli Lilly and Co., Indianapolis, Ind., U.S.A.: glucagon.

Behringwerke A.G., Marburg/Lahn, G.F.R.: bovine serum albumin, dried purified.

Packard Instruments Co., U.S.A.: Insta-Gel^R scintillation

fluid.

Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.: Chromar-Sheet^{R} 500.

Highly purified natural cholecystokinin-pancreozymin (CCK-PZ, pancreozymin, activity 3000 Ivy Dog Units per mg) has been kindly donated by Dr. V. Mutt, Karolinska Institute, Stockholm, Sweden.

Dr. M. Ondetti, The Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A., has donated the synthetic secretin (Pansecrin^{R}, activity 3000 Clinical Units per mg) and the pancreozymin C-terminal octapeptide (pancreozymin octapeptide, activity 16,000 Ivy Dog Units per mg).

Phosphatidylcholine, labeled with [^{14}C]-octadecenoic acid in the 2-acyl position, 3×10^{8} cpm per mmol, and phosphatidylcholine, labeled with [^{3}H]-hexadecanoic acid in the 1-acyl position, 34×10^{6} cpm per mmol, are a generous gift of Dr. H. Van Den Bosch of the Laboratory of Biochemistry, The State University, Utrecht, The Netherlands. By incubation with purified snake venom phospholipase A_2 (Sigma Chemical Co., St. Louis, Mo., U.S.A.) it has been shown, that 20% of the labeled fatty acid in the [^{3}H]-phosphatidylcholine is present in the 2-acyl position. With help of thin layer chromatography it has been shown, that the [^{14}C]-phosphatidylcholine contains 0.9% [^{14}C]-lysophosphatidylcholine, while the [^{3}H]-phosphatidylcholine contains 3.3% [^{3}H]-lysophosphatidylcholine, 0.1% [^{3}H]-free fatty acid and 2.4% [^{3}H]-phosphatidylethanolamine.

All other reagents were commercial preparations of the highest obtainable purity.

CURRICULUM VITAE

De auteur van dit proefschrift, W.J.M.J. Rutten, werd op 11 januari 1942 geboren te Nederweert. Na het behalen van het eindexamen gymnasium β aan het Bisschoppelijk College "St. Joseph" te Weert in 1959, ving hij in hetzelfde jaar aan met de studie in de farmacie aan de Rijksuniversiteit te Utrecht. Na in december 1963 het kandidaatsexamen te hebben afgelegd, behaalde hij in september 1967 cum laude het doctoraalexamen. De apotekersexamens, deel I en II, legde hij respectievelijk af in maart 1968 en maart 1969. Gedurende zijn studie leverde hij vanaf 1 augustus 1964 als kandidaat-assistent en vanaf 1 oktober 1967 tot 1 april 1969 als doctoraal-assistent een bijdrage in het onderwijs aan studenten in de farmacie. Van 1 april 1969 tot 1 januari 1973 was hij werkzaam als wetenschappelijk medewerker bij het Laboratorium voor Biochemie van de Medische Fakulteit van de Katholieke Universiteit te Nijmegen. Hier leverde hij een bijdrage in het onderwijs aan medische, tandheelkundige en chemische studenten en voerde tevens het hier beschreven promotie-onderzoek uit. Vanaf 1 januari 1973 is hij als ziekenhuis-apoteker werkzaam te Enschede.

STELLINGEN

I

Bij de keuze van een methode voor de bepaling van de aktiviteit van cyclisch AMP phosphodiesterase dient, in het bijzonder wanneer weinig gezuiverde preparaten onderzocht worden, rekening gehouden te worden met de mogelijkheid, dat het produkt van de enzymatische reaktie tijdens de inkubatie verder gemetaboliseerd wordt.
Dit proefschrift, Hoofdstuk IV

II

De observatie van Darrow-Brown et al., dat in weefsel van chemisch geinduceerd mammacarcinoom bij ratten een zeer hoge adenylaat cyclase aktiviteit aanwezig zou zijn, moet zeer waarschijnlijk geweten worden aan de door de auteurs gebruikte inadequate bepalingsmethode.
Darrow-Brown, H., Chattopadhyay, S.K., Spjut, H.J. en Spratt, J.S., (1969), Biochim. Biophys. Acta, 102, 372-375
Bär, H.-P., Biochim. Biophys. Acta, 321, 397-406

III

Een bijzonder hoge kapaciteit van cyclisch AMP phosphodiesterase in een weefsel kan in een aantal gevallen een mogelijke verklaring vormen voor het falen van cyclisch AMP en/of phosphodiesterase remmers om een hormoon-effekt na te bootsen
Dit proefschrift, Hoofdstuk II en V

IV

De bepaling door Robberecht et al. van de kinetische parameters K_m en V_{max} van cyclisch AMP phosphodiesterases met verschillende K_m in rattepancreashomogenaten d.m.v. een Lineweaver-Burk plot levert onjuiste waarden, omdat de auteurs de gemeten reaktiesnelheden niet splitsen in de bijdrage daarin van de afzonderlijke enzymen.
Robberecht, P., Deschodt-Lanckman, M., De Neef, P. en Christophe, P., (1974), Eur. J. Biochem., 41, 585-591
Dit proefschrift, Hoofdstuk V

V

Bij experimenten met pancreas preparaten in vitro dient er rekening mee gehouden te worden, dat de resultaten in sterke mate beinvloed kunnen worden door de destruktieve werking van de zelfs bij lage temperatuur aktieve, in de pancreas aanwezig zijnde, lipolytische enzymen.
Dit proefschrift, Hoofdstuk VII

VI

Bij de interpretatie van de resultaten van experimenten, waarin de calcium-uitwisseling in pancreas-coupes wordt onderzocht, verzuimen Case en Clausen een onderscheid te maken tussen uitwisseling over de serosale en luminale membranen.
Case, R.M. en Clausen, T., (1973), J. Physiol., 235, 75-102

VII

Het is niet onwaarschijnlijk, dat een belangrijk deel van de door Neufeld et al. gevonden calcium binding in fotoreceptor membraansuspensies veroorzaakt wordt door de aanwezigheid van mitochondrien.
Neufeld, A.H., Miller, W.H. en Bitensky, H.W., (1972), Biochim. Biophys. Acta, 266, 67-71

VIII

De onzorgvuldigheid van de redaktie van dat gedeelte van de door de Stichting 'Eurotransplant" uitgegeven handleiding, waarin de vloeistof voor preservatie van donornieren (Collins vloeistof 3) beschreven wordt, betekent een potentieel gevaar voor de ontvangende patient.
Eurotransplant Manual, First Edition, Eurotransplant, University Hospital, Leiden
Collins, G.M., Bravo-Shugarman, M. en Terusaki, P.I., (1969), Lancet, II, 1219-1222

IX

Bij de uitvoering van plannen tot schaalvergroting in de ziekenhuisfarmacie dient de positie van de intramurale apotekers binnen de ziekenhuizen gehandhaafd te blijven.

X

Voor het funktioneren van de farmacie volgens moderne maatstaven is het wenselijk, dat er een opleiding op HBO-niveau voor farmaceutisch hulppersoneel ingesteld wordt, zodat de te grote afstand tussen academisch en op MBO-niveau geschoolden overbrugd wordt.

XI

Hoewel chemische contraceptiva niet als verpakte geneesmiddelen beschouwd worden, verdient het aanbeveling, dat de overheid dergelijke middelen niet op de markt toelaat, voordat zij op hun onschadelijkheid onderzocht zijn.

XII

De ontwikkeling van de psycho-sociale zorg in de kindergeneeskunde kan, mede doordat er aandacht geschonken wordt aan de preventie van psychische problemen, een bijdrage leveren aan een vermindering van de kosten van de gezondheidszorg.
Wolters, W.H.G., (1973), Maandblad Geestelijke Volksgezondheid, 78, 327-341

XIII

Het is wenselijk, dat ten behoeve van het onderwijs in de moderne talen leermateriaal ontwikkeld wordt, waarin de positieve elementen van de strukturele en de cognitivistische methodes van taalverwerving geintegreerd zijn.

XIV

Het mag op zijn minst een twijfelachtige opvatting genoemd worden, dat verbindingen als askorbinezuur en ijzer(II)-sulfaat geen farmakologische eigenschappen zouden hebben.
Van der Meer, J., (1974), Ned. T. Geneesk., 118, 548-549

XV

Hallelujah, niets aan de handah!
Reve, G., (1974)

Nijmegen, 20 juni 1974 W.J.M.J. Rutten